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THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE

II. The Cyclic Changes in the Ovarian Soma during Gestation

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Introduction

In an introductory article on the reproductive cycle of *Neotoca bilineata*, an account was given of the breeding cycle of the female as observed in the laboratory (Mendoza, 1939). In this second paper of the series, a description is given of the changes which the ovarian soma undergoes during gestation. Previous articles on the reproductive cycles of viviparous teleosts usually have failed to give complete accounts of the somatic cycle although the later papers on *Fitzroyia lineata* (Scott, 1928), *Xiphophorus helleri* (Bailey, 1933), and *Cymatogaster aggregatus* (Turner, 1938) are notable exceptions. It is the purpose of this account first, to supplement the original, brief description given by Turner in 1933 and second, to contribute to a better understanding of the marked changes which a typical viviparous ovary undergoes during gestation. The discussion includes (1) a brief reference to the gross morphology of the ovary, (2) the general ovary-embryo relations, and (3) a detailed account of the cyclic changes in the ovigerous folds, the epithelium, the free cellular elements, and the blood supply.

Materials

The ovaries used in this investigation are from females whose breeding cycles were closely followed in the laboratory (Mendoza, 1939). A complete series was obtained ranging from virgin and non-gravid ovaries through all stages of gestation to an ovary undergoing involution three hours after the birth of the young (Table I). In the discussion to follow, ovaries will be referred to, not by the number of the female, but by the length in millimeters of the contained young (3.5, 7.2, etc.). Thus the emphasis is placed not on any one individual female but on the conditions typical of that particular stage of gestation. Ovaries earlier

than 1.5 will be designated appropriately. Most of the larger ovaries were sectioned after the young had been removed from one side and measured; the smaller ovaries were sectioned intact. The fixing fluid used most was that of Bouin although Zenker's, Fleming's, and corrosive sublimate were used for special fixations. The alcohol-xylol series was used almost entirely for dehydrating and clearing. All embedding was done in paraffin and serial sections of each ovary were made at a thickness of 10 micra. Iron hematoxylin was used as the standard stain with orange G as the principal counterstain although eosin Y and acid fuchsin were used occasionally. The stains of Mallory, Van Giesen, and a modified Bielschowsky (Foote and Menard, 1927) were used to

TABLE I

The complete series of ovaries sectioned and examined during the study of the somatic cycle of the ovary.

Number of ovary	Stage of embryos	Number of ovary	Stage of embryos
No. 3	Ovocyte (non-gravid female)	No.14	3.0 mm.
4	" " "	44	3.5 "
6	" " "	8	4.5 "
15	" " "	12	4.5 "
20	" " "	23	6.0 "
56	" " "	27	6.0 "
74	" " "	9	7.0 "
65	blastodisc	11	7.2 "
22	early segmentation	1	9.0 " (birth of young)
73	" "		
19	late segmentation	66	3 hours after birth of young
47	1.5 mm.*		
16	2.7 mm.	54A	virgin female
		54B	" "

* The numbers in millimeters represent the average length of the contained young in one side of the ovary.

differentiate connective tissue whereas Fleming's fixation identified fat and Mayer's muci-carmine was used as the test for mucin. All photomicrographs were taken with a Zeiss microscope and a Reflex-Korelle camera.

GROSS MORPHOLOGY

The ovary is a single structure approximately 5–10 mm. long, colorless, spindle-shaped, and with no external lobulations; it lies in the extreme dorsal portion of the pleuro-peritoneal cavity, attached by a short mesovarium along the median dorsal line of the coelom. As is true of all the viviparous teleost ovaries known to the writer, the gonad and its single oviduct are continuous, with no marked line of demarcation be-

tween the two. The structural relations within the ovary are fundamentally similar to those present in other strictly viviparous teleosts, namely, an external sac to which are attached in a diverse manner lobulated folds bearing the germinal tissue. In *Neotoca*, the ovary is divided into two longitudinal chambers by a heavy perforated septum in the median sagittal plane. Each chamber in turn is filled almost completely by a large, lobulated, ovigerous fold attached by a narrow strip of tissue along the dorso-lateral wall of the ovarian sac (Fig. 1). The internal surface of the ovarian wall is characterized in turn by low, longitudinal folds. In the region of the oviduct, the median septum and the ovigerous folds are absent; only the ridges or folds along the wall remain, accentuated in size (Fig. 9).

The tissues involved in the ovary are few. A continuous internal epithelium lines the ovarian cavity completely investing the ovigerous folds, the ovarian wall, and the median septum (Plate I). A loose stroma of connective tissue under the epithelium forms the bulk of the ovigerous folds (Figs. 1–6, 11, 12), the septum, and is continued around the ovarian wall as a narrow layer. Forming the external wall of the ovary is a heavy layer of circular muscle richly interspersed with collagenous connective tissue fibers (Figs. 1, 21); the muscle layer in turn is invested by a serosa. Four pairs of large blood vessels surrounded by very heavy coats of muscle and connective tissue course along the wall of the ovary at the ends of the median septum and at the base of each ovigerous fold (Fig. 1). Smaller branches ramify to a rich, subepithelial plexus of capillaries (Fig. 10). A complete description of the germ cells which occur within the stroma of the folds will be reserved for a later paper.

Embryo-Ovary Relations

The ovocytes develop within follicles in the ovigerous folds (Fig. 1), are fertilized while still within the follicle, and are expelled immediately into the ovarian lumen (Fig. 2). Consequently, the entire developmnt of the embryos occurs within the lumen of the gonad proper, within the limited space left between the ovigerous folds and the ovarian wall. Although no special relations are established between the embryo and the ovary as in *F. lineata* where processes of the ovarian tissue actually penetrate into the branchial chamber of the embryos, in *Neotoca* the young always remain in close proximity to the ovarian folds. During gestation, the ovary undergoes radical modifications which again subside with birth of the embryos. Accompanying these changes, the young likewise become modified, developing huge absorptive processes that are resorbed at time of birth (Turner, 1933, 1937; Mendoza, 1937). In view of

the scarcity of yolk in the ova, it has been assumed that the marked modifications on the part of the ovary and the embryo are to supply the necessary nutritional and respiratory relations between the two. It is, therefore, with the cyclic somatic changes undergone by the ovary during gestation that the present paper is concerned.

Cyclic Changes

The most conspicuous changes in the ovary are: (1) an increase in the dimensions of the ovary, (2) a swelling of the ovigerous folds, (3) a marked secretory activation of the internal epithelium, (4) an increase in certain wandering cells of a secretory nature, and (5) an increase in the vascularity of the ovary.

Size

Although a resting ovary averages approximately 1.5 mm. in diameter (Fig. 1), a gravid ovary at term measures 10–15 mm. in diameter (Fig. 7), occupies the greater part of the coelom and distends the abdominal

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EXPLANATION TO FIGURES

All figures are photomicrographs taken with a Zeiss microscope and a Reflex-Korelle camera.

Plate I

A series of cross-sections of ovaries, photographed under the same magnification and showing the gross changes of the ovary and young during gestation. This series is completed with Figs. 7–9 on Plate II. The scale of magnification for this series appears in Fig. 9 of Plate II.

Fig. 1. A non-gravid ovary. Clearly visible are: the outer muscular wall, the internal epithelium of the ovary, the median septum, one highly lobulated ovigerous fold on each side of the median septum, the paired blood vessels appearing especially distinct at the base of each of the ovigerous folds, and developing ovocytes which appear as round structures within the ovigerous folds. The cut in the wall is the incision normally made in most ovaries before fixation.

Fig. 2. An ovary with young in stages of germ-layer formation. One normal embryo appears just below the center of the ovary. The folds are slightly swollen.

Fig. 3. An ovary with young 1.5 mm. in length. The folds are swollen and the epithelium is starting to secrete. A section through the tail region of one embryo appears in the upper right.

Fig. 4. An ovary with young 3.5 mm. in length. The folds are swollen and the epithelium is at its maximal point of secretory activity. One young occupies almost the entire left side of the figure.

Fig. 5. One-half of an ovary with young 4.5 mm. in length. The ovarian wall is more tenuous because of the growth of the embryos. Two young fill one-half of the ovarian lumen.

Fig. 6. A small section only of an ovary with young 6.0 mm. in length. The ovigerous folds are still swollen; two young are visible.

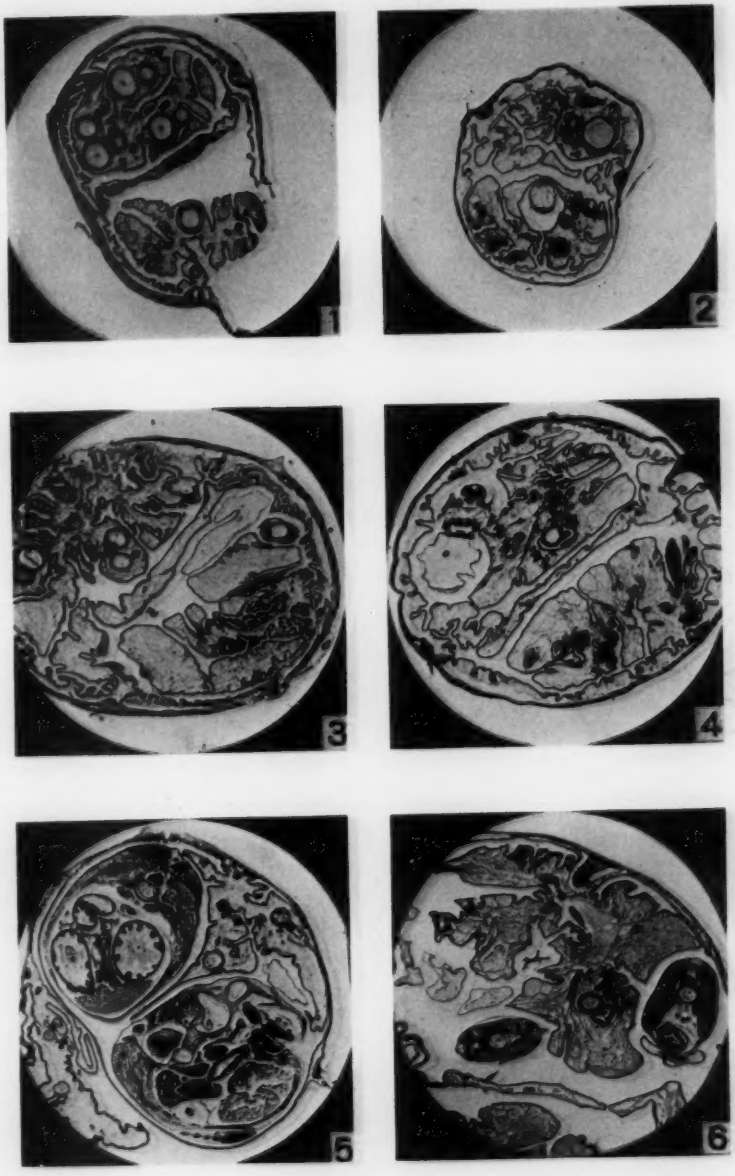


PLATE I

wall of the female far beyond normal proportions. Simultaneously, due to extreme expansion, there is a resulting decrease in the thickness of the muscular wall from an average thickness of 55 micra in a resting ovary (Fig. 21) to 5–10 micra in an ovary just prior to the birth of the young (Fig. 22), a decrease to almost one-tenth of the original thickness.

Ovigerous Folds

The ovigerous folds consist of a loose connective tissue reticulum completely invested by the internal epithelium of the ovary. Within the folds but adjacent to the surface lie the nests of developing ovocytes (Fig. 1). In the resting ovary, the fibers of the reticulum are loose, wavy, and branched (Fig. 11). The greater part of the reticulum is composed of thin delicate fibers; however, there occur occasional masses of heavy fibers scattered at random in the stroma and especially underneath the epithelium. Differential staining shows that the delicate fibers are of a collagenous nature whereas the heavy fibers not only are collagenous but in addition show a marked affinity for argyrophylic or reticular connective tissue stains. In the interstices of this tissue there occur scattered droplets of an oily nature and small quantities of a lymph-like fluid that coagulates upon fixation. In addition to the fluid bathing the reticulum there also occur many types of wandering cells to be described later. Although in the typical resting ovary the ovigerous folds are small, compact, and with but little contained fluid (Fig. 1), advance in gestation is accompanied by marked changes. The folds begin to

PLATE II

FIG. 7. A very small portion of an ovary with young 9.0 mm. in length and ready for birth. The ovigerous fold is collapsed and appears in the upper center of the figure. The two delicate structures in the center between the large embryos are sections of the absorptive processes of the young. The large black spots in these and other absorptive processes in the figure are blood vessels that supply the superficial capillary plexus of the processes.

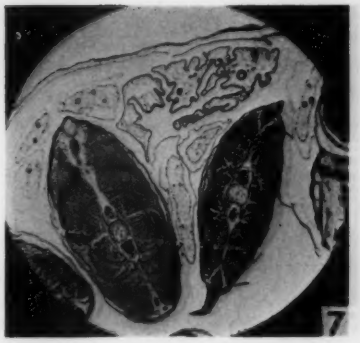
FIG. 8. An ovary three hours after the birth of young showing the nearly complete involution of the gonad. These and all preceding figures were taken under the same magnification and give an idea of the radical changes in size the ovary undergoes during gestation.

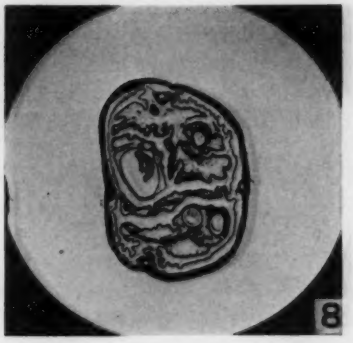
FIG. 9. A section of the oviduct. The scale represents the final magnification for all figures from 1–9.

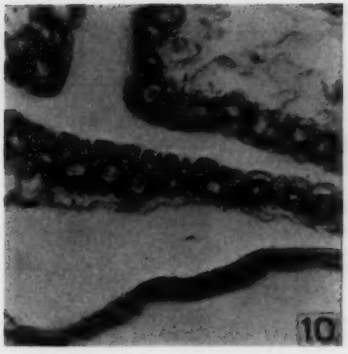
FIG. 10. An oil-immersion photograph showing the subepithelial capillary plexus in an ovary during the latter stages of gestation (7.0 mm). The black rings that show prominently in the lower section of epithelium are heavy connective tissue fibers that surround the capillaries. Some erythrocytes are visible within the vessels.

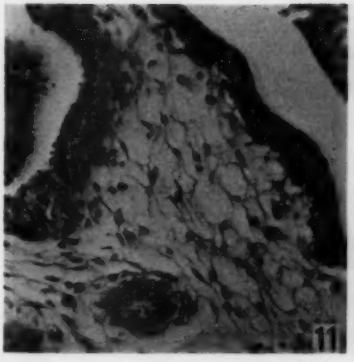
FIG. 11. A high-power view of a portion of an ovigerous fold in a non-gravid ovary.

FIG. 12. A high-power view of a portion of an ovigerous fold in a gravid ovary. The fold is swollen by the contained fluid. The reticulum of the stroma is stretched like a taut network. The scale for this and the preceding figure appears at the lower left.









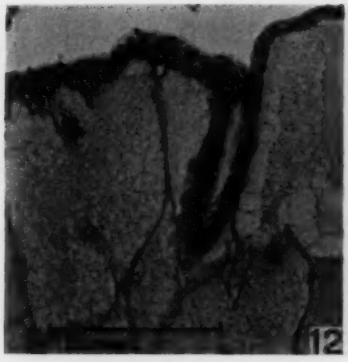


PLATE II

swell noticeably with a resulting turgidity caused by the increase of the contained fluid (Fig. 2); these changes become prominent when embryos reach stages of segmentation and germ layer formation. The maximal condition of swelling and turgidity of folds is reached between stages 1.5 and 6.0 at which time the folds usually are large, rounded, and seemingly full of a flocculent substance (Figs. 3, 4, 6). The pressure of the fluid may be so great at times that individual cells or an entire section of epithelium may become elevated from the surface of the folds. In addition, the reticulum usually becomes stretched like a taut net within the folds (Fig. 12). It is evident, consequently, that the increase in the size of the ovary during gestation is caused not only by growth of the embryos but also by the marked swelling of the ovarian tissues. Finally, in the last stages of gestation, the folds which are no longer rounded often become moulded in their contour by the presence of the large embryos; the folds actually have the appearance of being depleted or collapsed, their purpose apparently having been served. There is little or no coagulated fluid and but few wandering cells so prominent at other times (Fig. 7). Three hours after birth of the embryos (Fig. 8), the folds appear to all purposes like those in a normal non-gravid ovary. Although there is some variation in the time of appearance and disappearance of the swollen condition, the typical cycle is as explained above.

It will be recalled that this reticulum of connective tissue is continued as a narrow layer around the non-ovigerous wall of the ovary, between

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PLATE III

A series of photomicrographs showing the changes in the ovarian epithelium during gestation. The series is completed with Figs. 19 and 20 on Plate IV. All photographs are magnified equally, approximately 620 ×.

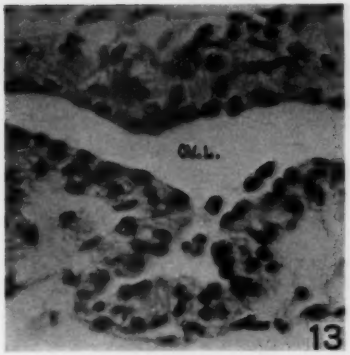
FIG. 13. A non-gravid ovary in which the epithelium is represented by cells with small, prominent nuclei and with little cytoplasm. Nuclei appear to intermingle with underlying fibrocytes. The ovarian lumen (*OV.L*) appears in the center.

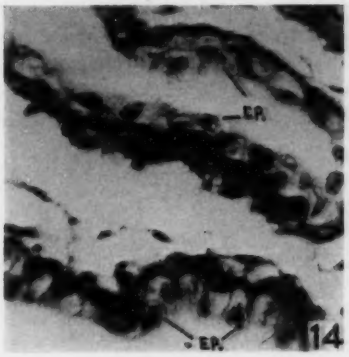
FIG. 14. An ovary with embryos in late segmentation stages. The epithelium (*EP*) is better defined than before since the cytoplasm is far more conspicuous. The cells are preparing for secretory activity.

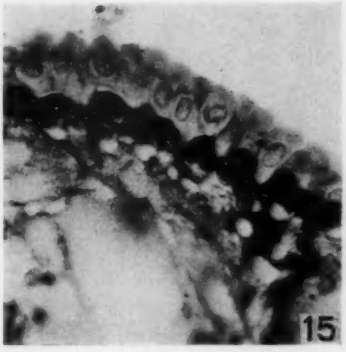
FIG. 15. An ovary with young 2.7 mm. in length. The cells of the epithelium are tremendously enlarged and actively secreting.

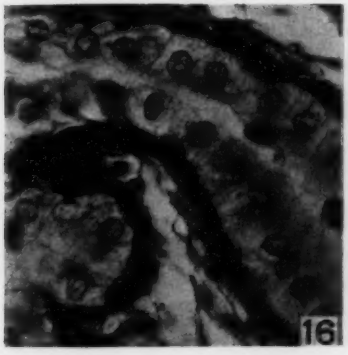
FIGS. 16 AND 17. Two views of the same ovary during maximal secretory activity. The young are 3.5 mm. in length. In both figures adjacent epithelia have become temporarily adhered to each other.

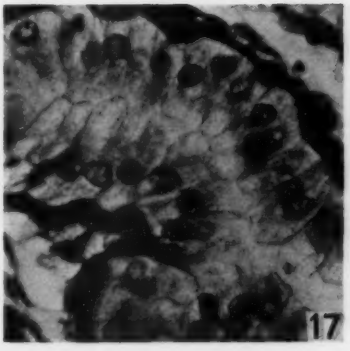
FIG. 18. A section of the median septum in an ovary with young 4.5 mm. in length. The epithelium appears to be decreasing in secretory activity; the cytoplasmic volume of the cells not only appears to be reduced but frequently there is a partial or complete collapse of the cell.











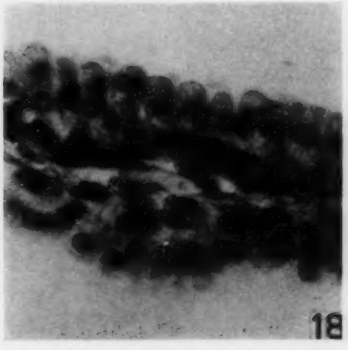


PLATE III

the internal epithelium and the outer muscular wall, and into the median septum. These regions, continuous with the stroma of the ovigerous folds, suffer identical changes (Figs. 3, 4). The increase in quantity of fluid with its resultant turgidity likewise affects these parts, all of them contributing to the spongy nature of the ovary during gestation.

Epithelium

The epithelium forming the inner surface is subject to as great a change as are the actual folds. In most resting ovaries, the epithelial cells have very prominent darkly-staining nuclei but with indefinite cell walls and very little cytoplasm (Fig. 13). The epithelium is flattened, almost embedded, against the subepithelial network of heavy connective tissue fibers identical with the similar ones in the ovigerous folds. This disorganized appearance usually is lost completely in stages of early segmentation for, whereas the cells may not be large, they become cuboidal in shape and regularly arranged at the surface (Fig. 14). During later stages, the cells of the internal epithelium of the ovary become columnar and greatly swollen (Fig. 15); they actually fuse with cells on adjacent ridges wherever they come into contact (Fig. 16). The cytoplasm is prominent now and well-defined having the typical appearance of that of a secretory cell. This enlarged condition of the epithelial cells, especially along the ovarian wall, is very characteristic of these and later periods of gestation. Between stages of 1.5 and 3.5, secretory activity reaches a maximum, simultaneous with the greatest turgidity of the ovigerous folds (Fig. 17). Whereas cell identification in resting ovaries is difficult (Fig. 13), during these later stages each cell stands out clearly, is tremendously enlarged, actively secreting, and frequently is disrupted at its distal end. The marked similarity between this epithelium and that of a mammalian uterus during the height of secretion is truly remarkable. This secretory activity continues later on though in a more abated fashion. On the whole, the cells now are more disorganized, collapsed, and some even are being sloughed off into the ovarian lumen; the general impression given is that of the abatement or cessation of secretory activity. This spent appearance reaches a maximum during late gestation (stages 6.0 and 7.2) when the cells show shrinkage and separation from adjoining cells (Fig. 10). In the last phase of gestation (9.0) the epithelium once more regresses to a thin cellular layer, giving no indication of secretory activity and flattened against the underlying subepithelial capillary plexus (Fig. 19). This condition is particularly true for the epithelium along the wall which has been extended so greatly that fibrocytes of the subepithelial connective

tissue, capillaries with their contained erythrocytes, and the epithelial cells themselves, to all appearances, form a continuous layer (Fig. 22). Finally, three hours after the birth of the young when the involution of the ovary is nearly complete, the epithelium again changes; it shows a definite attempt to readjust itself after the radical extension undergone during gestation. The cells now are small, with prominent, vesicular nuclei, devoid of practically all cytoplasm, and very loosely attached (Fig. 20). Many cells actually are superimposed; many others are being lost into the ovarian lumen. It is interesting to note that the large quantities of secretion elaborated during the early and middle stages of gestation now are completely lost. There is nothing left but the mere skeleton of the formerly enlarged cells which now apparently are in a state of physiological exhaustion.

This description of changes in the internal epithelium is characteristic of its entire extent, not only the investment of the ovigerous folds and of the median septum, but particularly of the ovarian wall.

Free Cellular Elements

In addition to the changes of the primary or permanent elements of the epithelium, there are equally important changes in the free cells or secondary elements that not only wander throughout the extent of the submucosa-like reticulum but also penetrate into the epithelium. It is evident that these cells contribute greatly to the swollen condition of the gravid ovary. The first cell to be described hereafter is called simply a secretory cell. It appears in almost all stages of early gestation, being present in the non-gravid ovary in variable numbers and increasing in number until the height of secretory activity in the epithelium. Then, the cell undergoes a marked transformation and decreases in number from stage 4.5 until the end of gestation. In stage 9.0 and in an ovary three hours after the birth of young, only few such cells occur. The tremendous increase in the number of these cells cannot be appreciated without numerical evidence. In earlier stages, 15–35 cells per section represent an average number but, in stage 3.5 (Fig. 24) an actual count of 798 cells in one typical section was made. The transformations undergone at this time are unique. An intracellular reticulum, attached to the a-centric nucleus, swells to a bursting point; it then collapses but the cell membrane remains intact with the contained fluid. The cell wall eventually ruptures and collapses, discharging the contents into the surrounding fluid. Since these cells wander throughout the ovary but eventually migrate into the epithelium, the elaborated contents are discharged mostly into the ovarian lumen. The identity and origin of the cell still are uncertain; nothing at all similar has been

found in the literature although absence of the cell from the blood stream precludes classifying it as a vascular element. With but few exceptions, its marked increase in numbers up to the time of maximal epithelial secretion and its radical transformation at that time leave little doubt of its contribution to the physiological state of the ovary. It is likely though not yet proven that the secretion elaborated by these cells may be of a nutrient nature.

A second cell type of importance is a granular cell of a variable nature. The nucleus is a-centric and picnotic; the large granules in the cytoplasm may be all basic, all acidophylic, or of a mixed character. The cell is amoeboid in nature and occurs throughout the ovary (Fig. 23). The conclusion that the cell is strictly a tissue granulocyte is drawn because of its complete absence from the blood stream. In resting ovaries it is present although the number varies somewhat; however, as the ovigerous folds approach maximal turgidity, these granular cells appear in maximal numbers. With the decrease of the secretory activity of the epithelium and the turgidity of the folds, these cells likewise diminish. In the latter stages of gestation, very few if

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PLATE IV

FIG. 19. The internal epithelium in the last stage of gestation preceding the birth of the young (9.0 mm.). The most prominent structure at the surface is the capillary plexus appearing as a series of rings. Stretched over the plexus appear three cells of the epithelium (arrows). Only the nuclei of the epithelium are prominent; the cytoplasmic content is negligible. A similar stage in which the capillary plexus appears more distinctly is represented in Fig. 10.

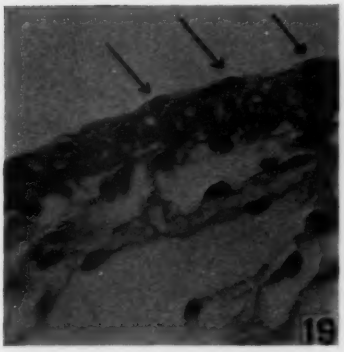
FIG. 20. The epithelium in an ovary three hours after the birth of the young. With the release of the tension on the ovary, the epithelium again becomes well-defined but the vast quantities of secretion and the cytoplasm have been discharged during gestation; only nuclei with a prominent chromatin mass remain. The cytoplasm forms a delicate sheath around the vesicular nucleus.

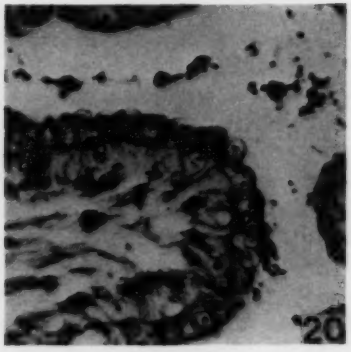
FIG. 21. The ovarian wall in a non-gravid ovary. The internal epithelium is at the upper edge of the figure; the muscular wall is at the lower edge. Although the nuclei of the muscle fibers are plainly visible, the collagenous connective tissue fibers do not show well.

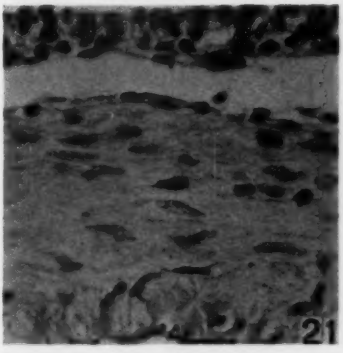
FIG. 22. The ovarian wall just before the birth of young. The magnification is the same as for the preceding figure. The tenuous epithelium again is at the top; the muscular wall is at the bottom. In the epithelium, three cells (arrows), few erythrocytes, and few fibrocytes can be distinguished. This epithelium appears as if under more tension than in Fig. 19 because in the preceding figure the epithelium is from the ovigerous folds, here, the epithelium is from the ovarian wall. The scale represents the magnification in Fig. 10 and all figures on Plates III and IV.

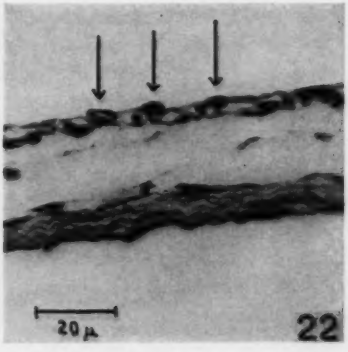
FIG. 23. A granular cell. The nucleus and some of the granules are in focus.

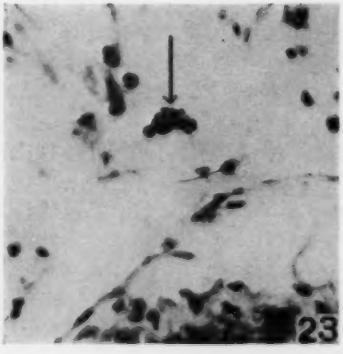
FIG. 24. Wandering secretory cells in the epithelium of an ovary actively secreting (stage 3.5 mm.). The darker bodies in the epithelium are secretory cells partially out of focus. The secretory cell identified by an arrow shows the details of the cell membrane, the picnotic nucleus at one end of the cell, and the intracellular reticulum attached to the nucleus.











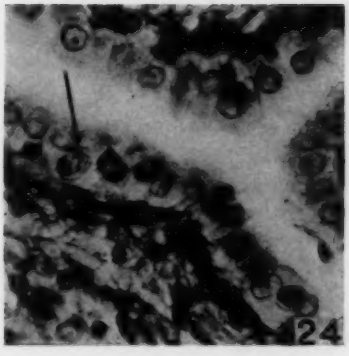


PLATE IV

any of these cells can be distinguished. Further evidence that these cells occur chiefly during the swollen condition of the ovary lies in the presence of the cells in large numbers in two virgin ovaries, ovaries that were in a very swollen condition. The significance of these activated virgin ovaries will be considered later. Whereas the secretory cells penetrate into the epithelium in large numbers, these cells seldom have been seen in the epithelium proper. That these cells accompany or contribute to the spongy condition of the ovary is verified further by actual disintegrating cells and the discharge of their contents into the surrounding medium. The secretion phenomenon through the solution of the huge granules is not improbable since a similar phenomenon has been reported before for eosinophilic cells (Jordan and Speidel, 1924).

Other cells of a primitive nature identified as stem cells from which are derived several of the free cellular elements also occur in large numbers. However, they follow the same cyclic behavior as followed by the two cells described above. Evidently the stem cells contribute to the swollen condition of the ovary only indirectly in so far as they give rise to other active secretory cells.

Blood Supply

Mention has been made already of the subepithelial vascular plexus. During resting phases, the epithelial blood supply is but little in evidence whereas in many ovaries there is a very rich blood supply to the maturing follicles. However, with the progressive development of the embryos, the general circulation increases and emphasis is placed, not on the follicular circulation but on the very prominent subepithelial plexus which remains most conspicuous throughout the rest of gestation (Fig. 10). It is likely that one of the most important rôles of this plexus is concerned with the respiratory gas interchange of the ovarian fluid.

Discussion

There can be no doubt that the ovary of *Neotoca* undergoes very definite cyclic modifications during gestation. The problem is whether this somatic cycle arises solely in response to the presence of the embryos or whether the cycle is inherent, appearing at stated intervals in the female. In other words, the problem is whether or not the ovary undergoes a periodic activation similar to that of the mammalian uterus. The problem arose when it was noticed that one or two of the resting ovaries differed from others in showing some evidence of swelling of the ovigerous folds and an increase in the secretory cells. Two known

virgin females then were killed for examination. Far from being in a resting condition, the ovaries were highly swollen, contained large numbers of granular cells, a typical resting epithelium, and but few secretory cells. This evidence further confirmed the presence of the granular cells only at a time simultaneous with the turgidity of the folds. Furthermore, these and other ovaries showed that the secretory cells may appear in variable numbers during early or resting stages but that the complete activation of the cells occurs only during the height of epithelial secretion. Lastly, the ovaries showed that they undergo partial activation even in the virgin condition without the inciting presence of the male or of embryos. In the first article of the series (1939) the writer showed the probable correlation of this swollen condition with the coloration of the female. At that time it was shown further that the coloration apparently was a cyclic phenomenon appearing at somewhat regular intervals. Hence the inference follows that, evidently, the ovary undergoes this somatic activation periodically. This activation may not be very precise in its rhythmic appearance; indeed, it would be very surprising if it were for it is likely that, judged from an evolutionary consideration, the assumption of the completely viviparous condition by this group of teleosts is quite recent.

The spongy condition of the gravid ovary is not entirely unique for this species since large " lymph spaces " in the ovarian tissues have been reported before for other forms: *Zoarces viviparus* (Stuhlmann, 1887); *Stygicola dentata* and *Lucifuga subterraneous* (Lane, 1903); and *Cymatogaster aggregatus* (Eigenmann, 1892; Turner, 1938). This spongy condition no doubt acts in a buffering capacity similar to that of an amniotic fluid; however, it is evident that it has even more vital functions, probably those of nutrition and respiration. Since the earlier studies of teleost viviparity, it has been suggested and assumed that the ovarian fluid must serve the embryos in just such a vital capacity. Actually, this assumption is not the result of chemical analysis but is largely a logical one based on the necessity for such a phenomenon in addition to the following observed facts: (1) there is an inadequate supply of yolk in the ovum (goodeids and others) to satisfy the needs of the growing embryos; (2) viviparous embryos generally develop vascular structures ideally suited for the absorption and interchange of substances (Turner, 1933, 1937); (3) there is an elaboration of vast quantities of secretion; and lastly, (4) there is the flooding of the ovary with a lymph-like fluid that coagulated upon fixation. Actually, it is likely that the only serious attempt to analyze the ovarian fluid in a viviparous ovary was made by Blake in 1867. He concluded the probable presence of an albumin compound with fat, phosphates, and iron. It is desirable that a thorough

chemical analysis of such an ovarian fluid be made for a viviparous teleost so that the assumptions made to date can be confirmed or rejected.

Lastly, the writer wishes to stress the marked parallel that exists between a viviparous teleost such as *Neotoca* and a placental mammal. In both forms there is the retention by the female of the developing embryos. In both forms the embryos are dependent on the female for the necessary nutritional and respiratory media because of the more or less complete absence of yolk in the ova and the retention of the developing embryos. In both forms there is a certain degree of similarity in the organ that houses the developing embryos, the uterus in one case and the ovary in the other. Structurally, both organs are similar in that both possess a highly secretory epithelium, a loose underlying connective tissue followed in turn by a heavy muscular coat and by a serosa. Both the uterus and the ovary of the respective forms undergo cyclic somatic modifications during periods of gestation. Both structures become highly vascular. Secretory activation and tumescence of the tissues occurs in both forms. The appearance of the decidual cells of mammals may have its counterpart in the occurrence of the secretory and granular cells in *Neotoca*. In both forms, highly vascular absorptive structures are developed by the embryos for the necessary nutritional and respiratory interchange; in one case, part of the intimate placenta is formed, and in the other, the embryos develop huge trophotaeniae (Turner, 1937) but remain free of the maternal tissues. And lastly, as was suggested in the previous article, there is the possibility that a period may occur in *Neotoca* similar to that of estrus in mammals. In the same way, it is possible that the activation of the ovary in *Neotoca* is a periodic, inherent one, similar to that of mammals. It is indeed unique that two forms, taxonomically as separated as are the teleosts and mammals, should become adapted in such a similar manner in response to a similar condition.

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THE RELATION BETWEEN OXYGEN CONSUMPTION AND RATE OF REGENERATION [1]

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Previous work (Barth, 1937, 1938*a*) showed that regeneration in *Tubularia* could be inhibited by placing the cut end of the stem in a glass capillary. A lowered O_2 tension was thought to be the cause of the inhibition and experiments in which the oxygen tension was varied showed that the rate of regeneration was closely dependent on the availability of oxygen. This relationship was also noted by Miller (1937), who found that the hydranth always appeared at the end where more oxygen was made available either by a higher oxygen tension or by circulating the sea water.

Further experiments by Zwilling (1939), in which the perisarc was removed from the middle of ligatured stems, showed that regeneration occurred on both sides of the perisarcal opening. This phenomenon could be interpreted to mean that the perisarc does not permit enough oxygen to reach the tissues to enable them to form a hydranth. The removal of the perisarc allows direct access to the oxygen of the sea water and starts the process of regeneration. If the foregoing experiments are to be explained on the basis of the concentration of oxygen at the tissues, then a study of the amount of oxygen consumed by the tissues is necessary. Some observations have been made by Child and Hyman (1926) on *Corymorpha* and Hyman (1926) on *Tubularia*. They found that the distal parts of stems consumed more oxygen than the proximal parts and further that young stems respired at a higher rate than old ones.

The experiments in this paper were designed to test whether: (1) the rate of regeneration of different levels of the stem is proportional to the rate of oxygen consumption at these same levels; (2) the regenerating portion of the stem uses more oxygen than the resting stem; and (3) whether the rate of regeneration of the stem is changed when the rate of oxygen consumption of a stem is increased or decreased.

[1] These researches were aided by a grant from the Rockefeller Foundation.

Methods

The stems of *Tubularia* were prepared from freshly collected material by cutting them off from the base of the colonies and selecting straight, unbranched stems of uniform thickness and healthy appearance. For the most part the more distal regions of the stem were used as these are free of parasitic growths which introduce an error in measurements of O_2 consumption. They were cut the desired length, care being taken to discard the region just adjacent to the hydranth as this region may exhibit a low rate of regeneration. The stems were then cut into halves, thirds, etc., according to the requirements of the experiment. The rate of regeneration was measured by taking the length of the primordium of the hydranth, the diameter of the primordium and the time at which the primordium becomes separated from the stem by a constriction. The rate of regeneration is then calculated as the volume of the primordium in μ^3 divided by the time in hours (Barth, 1938*b*). The units for rate of regeneration are $\mu^3/\text{hours} \cdot 10^5$.

The O_2 consumption was measured with Warburg manometers and the O_2 uptake calculated as the number of cubic millimeters of O_2 per hour per 10 mg. of dry weight. Weighings were made on a microbalance to 0.001 mg. In some of the earlier experiments the stems were merely selected of the same size and the oxygen uptake calculated for the mass of stems without weighing.

The O_2 Consumption of Parts of Stems

In these experiments the stems were cut into 2, 3, 4, or 5 parts and the rate of O_2 consumption determined. In some cases the rate of regeneration of the hydranth was also measured although previous experiments (Barth, 1938*b*) show that the rate falls off from distal to proximal region of the stem. The results have been calculated on the basis of $mm.^3$ O_2 used per hour per 10 mg. dry weight of the stem. Table I gives the results of using distal and proximal halves of stems. Since each half forms a hydranth at both cut ends, the rate of regeneration of the distal (oral, apical) hydranth and proximal (aboral, basal) hydranth of each half is measured. The table shows that the distal half consumes more oxygen than the proximal half (18.7 compared with 11.9) and the rate of regeneration of the distal half is greater (53.2 compared with 34.2). Likewise, in thirds of stems the rates of O_2 consumption and rates of regeneration are highest in the distal third, lower in the middle third and lowest in the proximal.

Table II records the rates of O_2 consumption of fourths of a stem.

Table III shows that the proximal fifth may consume as much O_2 as the third fifth of the stem, although since the stems were not weighed

TABLE I

Rate of oxygen consumption of parts of stems of *Tubularia*.
Rate = mm.^{3} O_2/hr./10 mg. dry weight.

Description of Stems			Oxygen Consumption					Rate of Regeneration μ^3/hrs. 10^5	
No.	Length	Region	Temp.	O_2	Time	Dry weight	Rate	Distal hydranth	Proximal hydranth
	mm.		°*C.*	*mm.*^{3}	*hours*	*mg.*			
20	7.5	distal half	19 ±.02	89.5	9.54	5.04	18.7	53.2	12.7
20	7.5	proximal half	19 ±.02	60.5	9.54	5.35	11.9	34.2	19.3
15	8	distal half	18.5±.02	112	13.42	3.512	23.8		
15	8	proximal half	18.5±.02	91	13.42	3.645	18.6		
20	5–6	distal third	19 ±.02	46.5	6.0	2.81	27.6	49.0	24.4
20	5–6	middle third	19 ±.02	41.0	6.0	3.06	22.4	34.4	17.4
20	5–6	proximal third	19 ±.02	31.4	6.0	3.36	15.6	23.6	9.6
10	5	distal third	18.5±.02	124	24				
10	5	middle third	18.5±.02	97	24				
10	5	proximal third	18.5±.02	89	24				

the results are not conclusive. This latter result agrees, however, with the experiments of Child and Hyman (1926), who found that the proximal third might sometimes use as much O_2 as the middle third. This observation correlates with the earlier observation of Child (1907) that the extreme proximal end sometimes regenerates as fast as higher levels of the stem.

It is clear then that there are regional differences in rate of O_2 consumption of the stem after cutting and that the rate of regeneration of the hydranth is roughly proportional to the rate of O_2 consumption.

TABLE II

Rate of oxygen consumption of parts of stems of *Tubularia*. In these experiments the proximal pieces were cut a little longer than the distal ones in an attempt to compensate for differences in diameter. As seen from the weight measurements they were cut a little too long and consequently are a little heavy. Rate = mm.^{3} O_2/hr./10 mg. dry weight.

No.	Length	Region	O_2	Time	Dry weight	Rate
	mm.		*mm.*^{3}	*hours*	*mg.*	
12	3	distal fourth	39.2	16.83	.977	23.8
12	3	second fourth	32.3		1.086	17.7
12	3	third fourth	30.0		1.190	15.0
12	3	proximal fourth	31.8		1.357	13.9
20	3–4	distal fourth	27.0	8.92	1.281	23.6
20	3–4	second fourth	22.8		1.517	17.0
20	3–4	third fourth	14.6		1.614	10.2
20	3–4	proximal fourth	21.2		2.023	11.7

Changes in the Rate of O_2 Consumption during Regeneration

When the values for O_2 consumption are plotted against time as in Fig. 1 the curve is S-shaped, indicating that as the regeneration process proceeds it requires O_2 at an increasing rate reaching a maximum and then falling off in the later stages of regeneration. The rate is highest from about seven to sixteen hours and it is during this period that the size of the primordium is determined (Peebles, 1931). These changes in rate are observed only in the case of short (2–4 mm.) stems.

The S-shape of the curve is lost or almost lost when the data from long (8–15 mm.) stems are plotted (Fig. 2). Here we find that the rate of O_2 consumption is almost constant throughout the period of regeneration. The interpretation given is that the O_2 consumption of

TABLE III

Oxygen consumption of fifths of stems of *Tubularia*. Figures are for total amount of oxygen in mm.^{3} O_2 consumed at time indicated.

No.	Length	Region	7 hours	26 hours	37 hours
15	2–3 mm.	distal fifth	31.4	125	162
		second fifth	23.0	104	140
		third fifth	18.3	95	125
		fourth fifth	16.8	83	108
		proximal fifth	22.0	94	122
			6.16 hours	17.33 hours	24.75 hours
17	2–3 mm.	distal fifth	17.3	52.0	63.0
		second fifth	14.6	41.5	51.0
		third fifth	12.4	38.9	49.5
		fourth fifth	13.3	36.6	48.5
		proximal fifth	11.8	34.4	46.0

the resting stem is so high that, in long stems where the regenerant comprises only about 10 per cent of the stem, the changes in rate caused by the regenerant are not noticeable. On the other hand in short stems, where 50 per cent of the stem may be regenerating tissue, the changes in rate during regeneration are more easily detected.

The Rate of O_2 Consumption and Rate of Regeneration at Varying O_2 Tension

The experiments of Miller (1937) and Barth (1937, 1938) indicated clearly that the rate of regeneration depended on the oxygen tension. In the following experiments the O_2 uptake was determined by placing the stems in different gas mixtures in the manometer vessels for most of the period required for regeneration. Then the stems were removed

and the rate of regeneration measured. Table IV gives the results. The rate of oxygen consumption falls from 5.4 in O_2 to 1.6 in N_2, while the rate of regeneration decreases from 177 to 0. It is clear that as the O_2 supply is reduced the O_2 consumption of the stem falls off and the rate of regeneration is decreased.

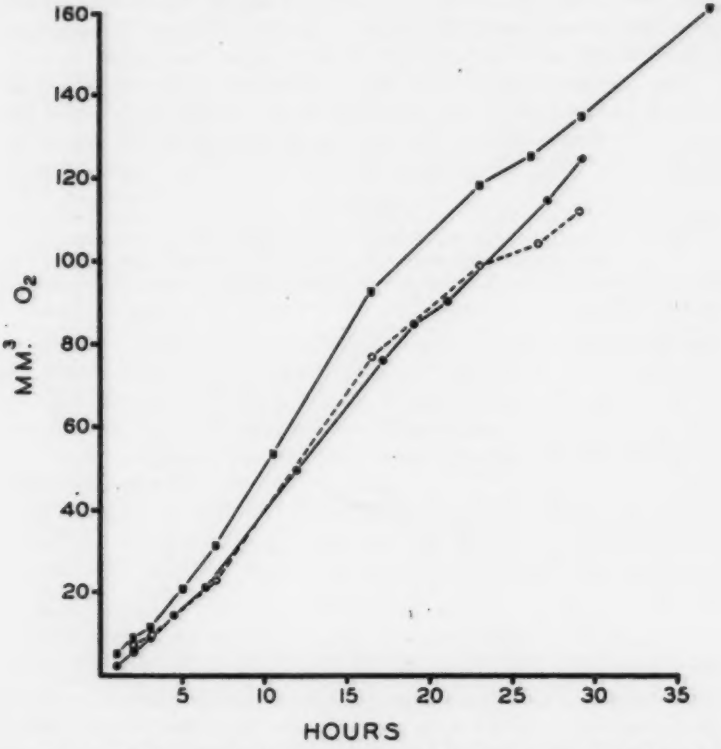

FIG. 1. Total amount of oxygen consumed by short stems, plotted against time. Squares give data from distal pieces of stems 2–3 mm. in length. Open circles are for more proximal pieces 2–3 mm. in length. Solid circles are for distal pieces 4 mm. in length. Hydranths are fully formed at 30 hours.

That the process of regeneration is closely dependent on the O_2 supply is shown by a comparison of the behavior of stems in the Warburg manometers and in open dishes. While stems never regenerate hydranths when ligatured at both ends and kept in open dishes, as many as 50 per cent of stems will regenerate hydranths if ligatured and shaken in the manometers with air. Thus, by keeping a high O_2 tension at the

surface of the perisarc, enough O_2 penetrates to start the process of regeneration. The O_2 consumption of these ligatured stems is about the same as for stems which have open ends (Table V). A similar result was obtained by Miller (1937) in comparing regeneration of ends in circulating sea water and standing sea water. More hydranths regenerate when the ends of the stem are bathed with circulating sea water.

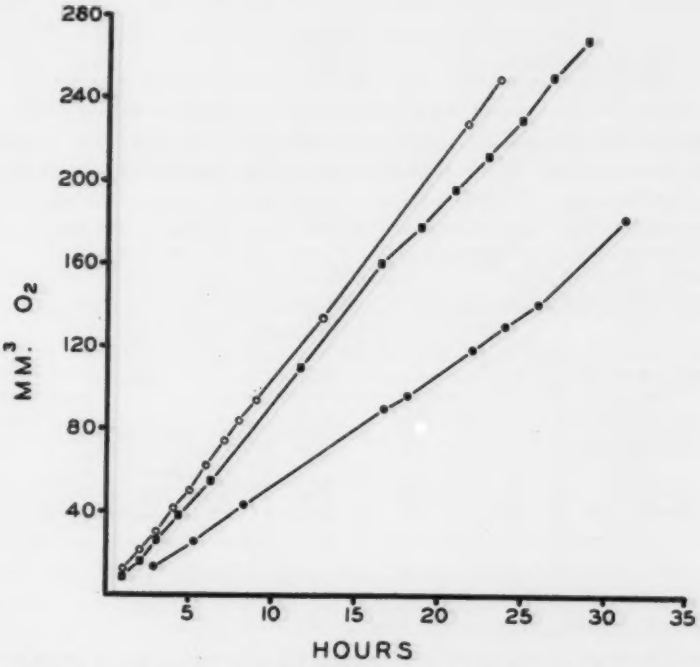

FIG. 2. Oxygen consumption of long stems, plotted against time. Squares = 12 mm. stems; open circles = 10 mm. stems; solid circles = 10 mm. stems. Hydranths fully formed at 30 hours.

Comparison of Rates of O_2 Consumption of Regenerating Stems with O_2 Consumption of Non-regenerating Stems

The rate of oxygen consumption of the regenerant itself must be only slightly greater than that of resting tissue. Attempts to measure the O_2 consumption of the regenerant itself were not very successful. The first attempt was made by ligaturing the stems and comparing the

TABLE IV

Rate of O_2 consumption and rate of regeneration at different O_2 tensions. Gas mixtures passed through vessels for 15 minutes with shaking. Twenty-seven stems (3 mm. in length) having a wet weight of 14 mg. were used for each gas mixture. Temperature 18.45 ± 0.02° C. Oxygen consumption measured for 28.75 hours. Stems then removed and the size of the hydranth measured.

Oxygen mixtures	Rate of regeneration μ^3 hrs. 10^5	Oxygen consumption mm.^{3} O_2/hr./14 mg. wet weight
O_2	177.0	5.4
air	155.0	4.9
1 vol. air to 5 vol. N_2	22.4	3.6
N_2	0	1.6

oxygen consumption of these stems with normal stems. The results are given in Table V. In both experiments the ligatured stems exhibited about the same O_2 consumption as non-ligatured stems. In the first experiment the ligatured stems did not form hydranths yet they consumed about the same amount of oxygen as those which did. The second experiment is complicated by the fact that regeneration occurred even in the ligatured stems.

TABLE V

Comparison of oxygen consumption of ligatured stems with non ligatured stems. Rate = mm.^{3} O_2/hr./10 mg. dry weight.

Description of stems	No.	Length	Rate	Remarks
		mm.		
Ligatured	7	12	31.4	No regeneration
Nonligatured	7	12	30.4	5 distal hyranths 1 proximal hydranth
Ligatured	10	10	38.4	50 per cent regeneration
Nonligatured	10	10	35.0	100 per cent regeneration

The second method of determining the rate of O_2 consumption of the regenerant consisted in a comparison of the oxygen uptake of a whole stem with that of its parts. Thus in Table VI, first experiment, 24 stems 12 mm. long were selected and 12 were cut into 4 pieces and the oxygen consumption of the fourths were measured. The remaining 12 were placed in a manometer vessel at the same time for comparison. In the case of the stems cut into fourths there are eight regenerating ends, while in the whole stem only two. The expectation was a more rapid rate of O_2 consumption with four times as many regenerants. However, Table VI shows that the O_2 uptake is about the same in the whole stem as in the sum of its parts.

Neither of these methods shows a measurable difference between the

amount of O_2 consumed by the regenerant and resting stem. However, the curves for O_2 uptake of long stems (Fig. 2) can be interpreted on the basis that in long stems the regenerant is small in comparison with the resting stem and thus any variation in O_2 uptake caused by the regenerant could not be detected. The S-shaped curves for short stems where the amount of regenerant is relatively larger give evidence that the regenerant uses more oxygen than the resting stem (Fig. 1).

Discussion

These experiments support the idea that the tissues that exhibit the higher O_2 uptake regenerate faster than those that use less O_2 (Child and Hyman, 1926; Hyman, 1926). It might be argued that the regional differences in ability to regenerate caused the difference in rate of O_2

TABLE VI

Comparison of the rate of oxygen consumption of whole stems with the rate of oxygen consumption of parts of the stem. Rate = mm.³ O_2/hr./10 mg. dry weight.

Description of stems	No.	Length	Rate
		mm.	
Distal fourth	12	3	30.6
Second fourth	12	3	22.1
Third fourth	12	3	20.9
Proximal fourth	12	3	19.2
Whole stems	12	12	23.4
Sum of fourths			22.8
Distal half	20	7.5	18.7
Proximal half	20	7.5	11.9
Whole stem	20	15.0	14.8
Sum of halves			15.2

consumption. This is unlikely, as in long stems where the regenerating region forms only a small fraction (1/10 or less) of the resting stem the difference in O_2 consumption of distal and proximal halves is present. Since the greater part of these stems is resting tissue, the difference in rate of O_2 uptake must be due to this resting tissue and not to the regenerant. The regenerant would have to consume O_2 at ten or more times the rate of the resting stem in order to produce the observed difference in rate of O_2 consumption of proximal and distal halves. (Table I.)

However, since the regenerant consumes only slightly more O_2 than the resting stem, its oxygen consumption cannot account for the regional differences in O_2 uptake measured in distal and proximal parts of stems. Therefore, the gradient in oxygen consumption is an inherent character-

istic of the resting tissues at various levels of the stem. The evidence from varying the concentration of O_2 to which the tissues are exposed indicates that as the tissues increase their O_2 consumption they are able to regenerate faster.

It will be of interest in further experiments to see whether the O_2 consumption can be varied without changing the rate of regeneration and also whether the rate of regeneration can be changed without affecting the O_2 consumption. A word of caution is necessary here since in the sea urchin's egg it is quite possible to increase the rate of O_2 uptake of the unfertilized egg until it equals that of the fertilized egg without stimulating the egg to develop. Thus the rate of regeneration and the rate of O_2 consumption may be dependent upon two different processes which thus far are affected by the same treatment and conceivably some treatment might be found where either could be changed independently of the other.

Summary

The rates of oxygen consumption and the rates of regeneration of parts of the stem of *Tubularia* are greater in the distal levels of the stem than in the proximal levels.

The regenerating end of the stem consumes oxygen at a rate which is not much greater than the rate of the resting stem.

In different gas mixtures the rate of oxygen consumption of the stem and the rate of regeneration of the hydranth vary in the same direction.

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BUDS INDUCED BY IMPLANTS OF POSTERIOR NERVE CORD AND NEIGHBORING TISSUES INSERTED AT VARIOUS LEVELS IN CLYMENELLA TORQUATA

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It has been reported that when pieces of nerve cord and neighboring tissues from anterior levels of *Clymenella torquata* (region of segments 1–7) are inserted into various segments of host of the same species the organization of the resulting buds, which frequently develop, is apparently greatly influenced by the host (Sayles, 1940). The orientation of the implant is, however, an important factor. Anterior pieces implanted with their anterior ends in the coelomic cavity and their posterior ends exposed at the surface of the host very rarely produce buds showing definite head or tail characteristics. It was earlier shown (Sayles, 1939) that even though, under some conditions, the nature of the bud might apparently be determined by the host, the new material nevertheless organized so that the exposed part of the implant was in the ventral part of the base of the bud with its original external surface again on the surface.

Implants from the posterior region of donors (segments 13–18) have now been used in 514 operations. In 194 of these the posterior ends of the implants were exposed, while in 311 it was the anterior ends which were at the surface. In 9 early cases no attention was paid to the matter of orientation. All of this experimental work was carried on at the Marine Biological Laboratory, Woods Hole, during the summers of 1935–1939, inclusive. The living material was generously provided by the staff of the Supply Department of the Laboratory.

Results

For all of the following operations the hosts and donors were first narcotized in a chloretone solution made by adding 1 part saturated solution (in sea water) of chloretone to 9 parts sea water. As in previous experiments, the implants were inserted in the dorso-lateral parts of the segments.

In the following discussion the segments of origin of the implants will be given in each case, using the expression " implant 14–15," for

example, to mean "implant from segments 14 and 15 of the donor." Except where otherwise stated, the description in each case is of the final condition reached by the bud either at the time of preservation or a brief time before the bud was lost.

Posterior End of Implant Exposed

The results of the 194 cases where the posterior ends of the implants were exposed are summarized in Table I. Although a few of these implants included part of the twelfth segment, the implant always had at least half of the thirteenth segment present. Thus, in no case was the exposed tip of the implant from a level anterior to the middle of the thirteenth segment. One of the interesting points is that, even at the most anterior levels, no head bud resulted from these posterior implants.

Of the 7 tail buds in the first group, 3 were at the *first* segment. One of these buds (Fig. 1), implant 13–14, consisted of about 7 seg-

TABLE I

Results of implantation of posterior pieces with posterior end exposed.

Level of insertion segments	1–4	5–7	8	9	10	11	12	13–15	Totals	Percentage of total
Tail + extra part	2	4	1	3		1			11	21.6
Tail buds	5	6		3	7	4	3	3	31	
Indeterminate buds	7	13	1	3	1	2		1	28	14.4
No new material	21	18	2		5	4		1	51	26.3
Died within 10 days	19	29		5	8	2			63	32.5
Implant lost by host	9		1						10	5.2
Totals	63	70	5	14	21	13	3	5	194	100.0

ments showing only on the ventral side beyond the end of the implant. The anal segment was open dorsally. No setae were evident. Another bud, implant 15–16, was short, unsegmented, and with most of the anal segment missing. The small part of the latter present (probably ventral region) terminated in 3 cirri. The third bud (Fig. 2), implant 12–13, included 8 segments, 5 setigerous. The anal segment was open dorsally. On the right side of this segment there was a large, extra, conical mass which showed no evidence of differentiation.

At the *second* segment, one tail bud (Fig. 3), implant 13–14, developed. No setae were present but the possible existence of 6 or 7 segments was indicated by transverse furrows which were confined almost entirely to the ventral side of the bud. Only about one-quarter or one-third of the anal segment was present. This partial segment was at the end of the ventral side of the bud.

At the *third* segment, there was formed one tail bud (Fig. 4), implant 13–14. On the dorsal side there were 2 setigerous segments followed by a short, asetigerous region. Ventrally there was evidence of approximately 7 short segments but no neuropodial uncini. The dorsal half of the anal segment region was missing. Just proximal to this open terminal region there was, at the end of the dorsal half of the bud, a large, undifferentiated, extra part of about the same size as the partial anal cone.

At the *fourth* segment, there were formed 2 large tail buds, each with some of the dorsal part of the anal segment missing. One of these buds, implant 14, included 5 setigerous segments and probably 3 asetigerous ones. The partial anal collar, only about half of which was present, was flattened out into a fan-shaped structure. The other bud (Fig. 5), implant 13, consisted of probably 10 segments, 7 of which were clearly setigerous. In this case only a small part of the anal segment was missing. This segment and particularly the collar and cirri were longest in the mid-ventral region and gradually decreased in extent until they were completely missing mid-dorsally. In each of these cases the bud included the approximate number of segments which one might expect to occur if the determining factor were in the implant.

Of the 10 tail buds found in the region of segments 5–7, one, implant 15, was at the *fifth* segment. This bud was too weakly segmented, even after 30 days, for one to be able to estimate the number of segments included. Only about one-third (ventral part) of the anal segment was present. Sixteen days after the operation a small, club-shaped structure was noted for the first time near the base of the anal segment on the dorsal side. This extra part did not undergo any evident changes during the remaining two weeks which the worm lived.

At the *sixth* segment, 7 tail buds developed. One, implant 12–13, was short and unsegmented. It terminated in what appeared to be a double anal collar region. As this worm died 8 days after the first appearance of new tissue at the implant, no very satisfactory idea of the exact nature of the bud was obtained. Two of these buds, implants 14–15 and 15–16, were unsegmented masses of new material each terminating in a small, partial, anal segment open dorsally (see Fig. 6). Another bud, implant 13–14, showed evidence of possibly several weakly differentiated segments but no setae. The dorsal half of the anal segment was missing. The fifth of these buds, implant 13–14, apparently included about 8 segments, on the basis of constrictions evident only on the ventral side of the bud. Only the ventral part of the anal segment was present. Each of the 2 remaining tail buds formed at this level included an extra part. One of these buds (Fig. 7), implant 13–14,

consisted of at least 3 setigerous segments followed by an asetigerous region with the dorsal side of the anal segment region open. On this same side of the bud there was a dorsally directed tongue of material a short distance from the open part. The other bud (Fig. 8), implant 12–13, included 4 setigerous segments followed by an asetigerous region which included a partial anal collar, open dorsally. On the terminal

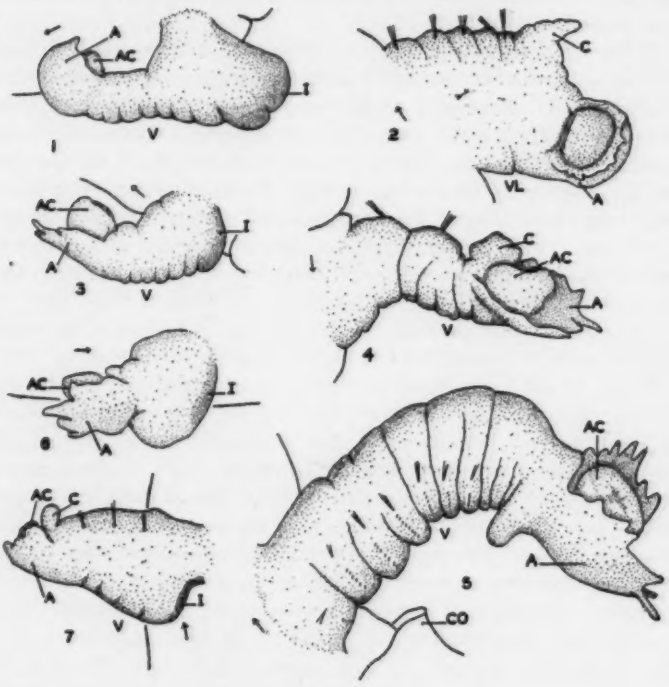

PLATE I

Arrow points toward anterior end of host in all figures.

All figures are camera lucida drawings and X22.

FIG. 1. Bud induced at first segment. Age, 18 days. *A*, partial anal segment; *AC*, anal cone; *I*, exposed part of implant; *V*, ventral side of bud.

FIG. 2. Bud induced at first segment. Age, 28 days. *C*, extra cone; *VL*, ventro-lateral region of bud.

FIG. 3. Bud induced at second segment. Age, 12 days.

FIG. 4. Bud induced at third segment. Age, 22 days. *C*, extra part.

FIG. 5. Bud induced at fourth segment. Age, 28 days. *CO*, collar of fifth segment of host.

FIG. 6. Bud induced at sixth segment. Age, 21 days.

FIG. 7. Bud induced at sixth segment. Age, 15 days. *C*, extra cone.

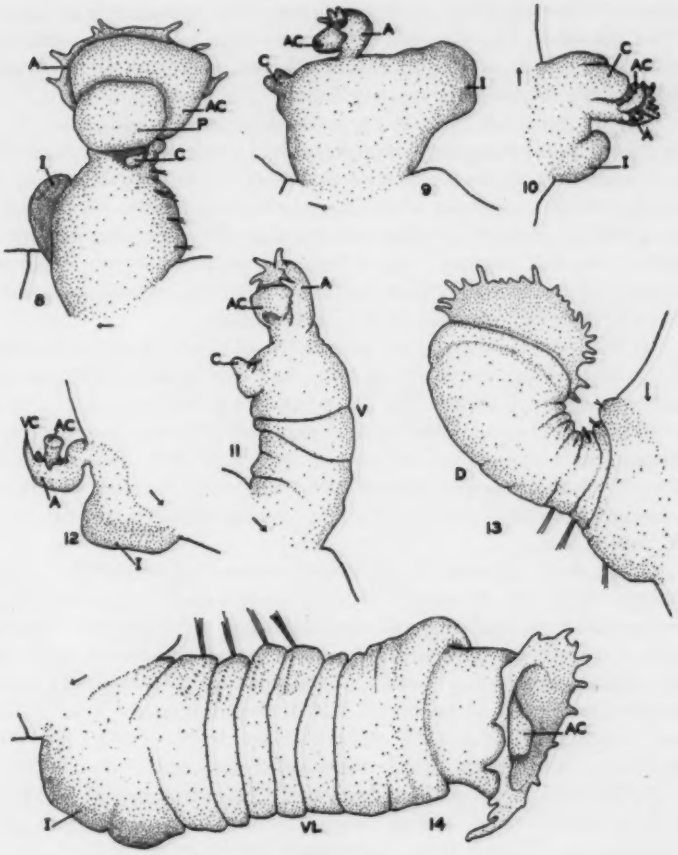

PLATE II

FIG. 8. Bud induced at sixth segment. Age, 23 days. *A*, partial anal segment; *AC*, anal cone; *C*, extra part; *I*, exposed part of implant; *P*, proboscis-like part.

FIG. 9. Bud induced at seventh segment. Age, 30 days.

FIG. 10. Bud induced at ninth segment. Age, 11 days.

FIG. 11. Bud induced at ninth segment. Age, 24 days. *V*, ventral side of bud.

FIG. 12. Bud induced at tenth segment. Age, 19 days. *VC*, long, mid-ventral, anal cirrus.

FIG. 13. Bud induced at tenth segment. Age, 27 days. *D*, dorso-lateral region of bud.

FIG. 14. Bud induced at tenth segment. Age, 33 days. *VL*, ventro-lateral region of bud.

region of the dorsal half of this bud there was a pointed elevation directed dorsally. On the side of this elevation toward the open anal segment there was a rounded part which had something of the appearance and the behavior of a proboscis.

One of the 2 tail buds at the *seventh* segment, implant 12–13, included 6 setigerous segments and probably 3 asetigerous ones, with only the ventral third of the anal segment present. The other bud (Fig. 9), implant 16, was made up of an unsegmented rounded mass possessing one group of notopodial setae and bearing: (1) near the end of the implant, an anal segment region only the ventral part of which was present; (2) on the side of the bud away from the implant, a somewhat irregular part without head or tail characteristics.

At the *eighth* segment, the one tail bud which developed, implant 12–13, was unsegmented, of medium size, and terminated in an anal segment region of which only the ventral third was present. On the dorsal side of the bud, near the anal segment, there was a long, slender process which showed no evidence of differentiation. This entire bud dropped off and was lost without being observed after the seventh day following the appearance of the first distinguishable new material at the implant.

At the *ninth* segment, 6 tail buds developed. Three of these buds, implants 12–13, 12–13 and 13–14, were unsegmented. Each of these 3 terminated in an anal segment the dorsal half of which was missing. Each of the other 3 buds included an anal segment region of which only the ventral portion was present. In addition each of these had a conical elevation at the end of the dorsal half of the main part. Two of these buds, implant 12–13 and 15, were unsegmented (see Fig. 10). The third (Fig. 11), implant 12–13, was weakly segmented.

At the *tenth* segment there developed 7 tail buds none of which had an extra part present. Three buds, implants 12–13, 14 and 15, were unsegmented. In each there was present less than half of the anal segment region, including several anal cirri. These 3 buds were all lost without any records being made concerning which side of the segment was missing. Another bud, implant 14, consisted of 5 or 6 weakly developed segments with the anal one open on one side. The part of this segment present was flattened out into a fan-shaped structure bearing 11 anal cirri. Another bud (Fig. 12), implant 16, was small, unsegmented, slender, and somewhat twisted. The dorsal side of the anal segment region was absent. The remaining 2 buds at this level had complete anal segments. One of these buds (Fig. 13), implant 13, consisted of probably 8 somewhat irregular segments of which the basal 5 were setigerous. The other bud (Fig. 14), implant 14–15, was large and

made up of 7 segments, 4 setigerous followed by 3 asetigerous. In this last bud there was the same number of segments as were found in the donor posterior to the source of the implant.

Of the 5 tail buds at the *eleventh* segment, two, implants 13–14 and 14–15, were weakly segmented but terminated in anal segment regions which were complete. In each case the anal collar was longest ventrally and bore a long mid-ventral cirrus. Another weakly segmented bud, implant 15–16, differed from the preceding in lacking the dorsal part of the anal segment. The dorsal half of the bud was truncate. The fourth

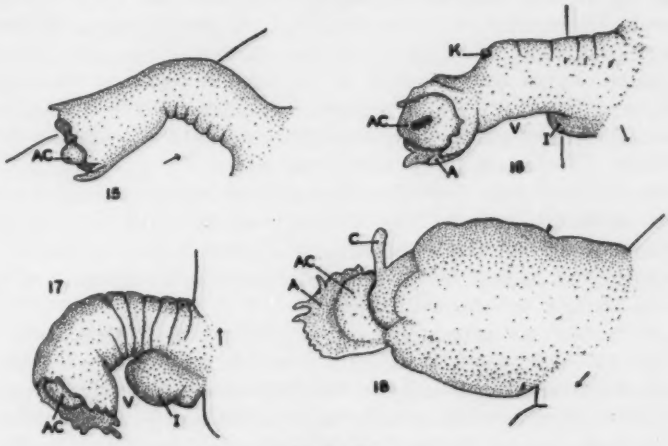

PLATE III

FIG. 15. Bud induced at eleventh segment. Age, 16 days. *AC*, anal cone.
FIG. 16. Bud induced at eleventh segment. Age, 16 days. *A*, partial anal segment; *AC*, anal cone; *I*, exposed part of implant; *K*, club-shaped extra part; *V*, ventral side of bud.
FIG. 17. Bud induced at twelfth segment. Age, 20 days.
FIG. 18. Bud induced at ninth segment. Age, 28 days. *C*, extra part.

of these buds (Fig. 15), implant 14–15, consisted ventrally of probably 7 segments. The anal segment and collar were complete. No setae were evident. A long mid-ventral anal cirrus was present, but the others were short. The last of these buds (Fig. 16), implant 13–14, consisted dorsally of at least 3 setigerous segments followed by an asetigerous region. Ventrally there was no evidence of segmentation. The anal segment was complete but with an anal collar which was very short on the dorsal side and without distinct cirri in that region. This anal segment, however, did not occupy the entire end of the bud but was associated mainly with the ventral half of this region. The dorsal half

of the bud ended in an oblique surface on the extreme dorsal part of which there was a small club-shaped structure. This was the condition 16 days after the implant operation. On the thirteenth day the dorsal third of the anal segment had been missing and the extra part had been present as a cone at the base of the open region. On the tenth day only about one-half of the anal segment had been present and the dorsal half of the bud had terminated in a large rolled edge.

At the *twelfth* segment 3 tail buds were formed. Two of these buds, implants 12–13 and 16–17, were unsegmented. Their anal segment regions were incomplete dorsally. The other bud (Fig. 17), implant 14–15, included, on its dorsal side, 7 segments of which the basal 4 were setigerous. On the ventral side, the implant occupied an unsegmented region corresponding to the basal 3 segments. Ventrally there were apparently 6 or 7 segments beyond the end of the implant. The anal segment was complete but shorter toward the dorsal side than ventrally. There was a long mid-ventral cirrus.

Of the 3 tail buds formed in the region of segments 13–15, 2 were at the *thirteenth* segment. One of these, implant 13–14, was a weakly segmented, long bud with a complete anal segment bearing 13 short cirri. The other bud, implant 14–15, consisted of probably 7 segments and had a complete anal segment and collar.

At the *fifteenth* segment, one tail bud, implant 14–15, developed. It consisted of 7 segments including a complete anal segment bearing a collar of ordinary height ventrally but shorter toward the dorsal region.

The indeterminate buds arising from the implants of posterior pieces did not differ essentially from those previously described as resulting from implants from anterior sources (Sayles, 1940). Three were weakly segmented but with their terminal regions rounded and showing no differentiation. Four were unsegmented, slender buds with rounded tips. Eight were small, undifferentiated cones. Three consisted of small amounts of new material which had developed beneath implant fragments. Ten were irregular masses of new material including one or more parts such as small knobs, one or two notopodia, or scattered neuropodial uncini.

Anterior End of Implant Exposed

The results of 311 operations in which the anterior ends of the implants were exposed are summarized in Table II. In no case was any part of the twelfth segment used in an implant. The most anterior level of an exposed end was, therefore, in the anterior part of a thirteenth segment. No head bud developed. Only one bud which it was

deemed safe to interpret as possibly a tail appeared. This one tail bud, implant 13 into segment 4, was of medium size, unsegmented, and with a terminal region which appeared to be a very weakly differentiated anal segment open on one side. This worm died soon after being examined on the fourteenth day after the implant operation.

Of the indeterminate buds, 14 were small cones or slender outgrowths, 2 were small irregular masses of new material, and 8 were new material which had pushed up the implants.

TABLE II

Results of implantation of posterior pieces with anterior end exposed.

Level of insertion segments	1–4	5–7	8	9	10	11	12	13	Totals	Percentage of total
Tail bud (?)	1								1	0.3
Indeterminate buds	4	1	8	4	5	1	1		24	7.7
No new material	34	23	29	24	18	1		5	134	43.1
Died within 10 days	54	38	26	21	4	2		1	146	46.9
Implant lost by host	1	3	1	1					6	1.9
Totals	94	65	64	50	27	4	1	6	311	99.9

Exposed End Unknown

Of the 9 operations for which no record was kept of the orientation of the implant, 4 hosts died within 10 days, 1 lived but produced no apparent new material, and 3 developed small cones or slender outgrowths. In one case, implant 12–13 into segment 9, there developed a large, unsegmented tail bud (Fig. 18) with the anal segment region open dorsally. At the base of this bud there was a single pair of notopodia. Near the open anal segment there was a slender, finger-like outgrowth projecting dorsally.

DISCUSSION

It has been shown (Sayles, 1940) that, following implantation of pieces of anterior nerve cord and neighboring tissues in *Clymenella*, buds of various types may develop. It seems that the determination of the type of bud to be produced in these cases may be controlled through an interaction of host and implant.

The data presented in Tables I and II show that there is a polarity of implants from posterior sources as definite as that shown by those from anterior regions of donors. Although 21.6 per cent of the 194

implants inserted with their posterior ends exposed produced buds with definite tail characteristics, only 0.3 per cent (1 case) of the 311 implants with anterior ends out produced similar results. The number of cases of indeterminate buds was also much smaller in proportion when the anterior ends of the implants were exposed. On the other hand, the percentage of cases with no evident new material was much greater in the group with the anterior ends of the implants exposed than in that where the posterior ends were out. This polarity is the reverse of that found among implants from anterior sources. The polarities exhibited by the pieces from the two sources are, however, both in accord with ordinary regeneration in *Clymenella* in which definitive buds can be produced only anteriorly in the region of segments 1–7 and only posteriorly at the levels of segments 13–22. Apparently the controlling factors which allow or bring about regeneration in one direction only in these anterior and posterior regions are present in these small implant pieces. This fact in itself does not mean, of course, that these factors are necessarily limited to the nerve cord and its vicinity.

It may also be noted that this polarity of implants gives us an interesting type of control experiment. For example, it might be thought that the occurrence of implant buds is due to a simple mechanical effect of the implants. It was reported (Sayles, 1939) that implanted pieces of intestinal wall or of dorsal body wall either failed to heal into the host or were gradually resorbed without the appearance of any evident new material. Admittedly there might be slight mechanical differences between these different types of pieces. The polarity of these nerve cord implants, however, gives us an excellent control experiment because the same small piece of tissue can induce a bud if oriented one way but not if inserted with the other end out.

It seems clear, therefore, that the implant bears the stimulating factor for bud formation. It also serves as the pathway along which new material migrates to the surface of the host. In the case of implants from posterior sources, as with anterior implants, the bud is organized along the longitudinal axis established by the implant. If the outer tip of the implant heals completely into the body wall of the host in such a way that it is not directed toward the surface of the body, the limited amount of new material which may appear at the surface is restricted in its organization to the possible formation of one or two notopodia or a few scattered neuropodial uncini. The data given in the present paper also add evidence that the orientation of the bud is controlled by the implant. In every case where information is available the implant is found at the base of the ventral side of the induced bud and the cuticle side of any exposed part of the implant is on the surface of the bud.

These experiments, in which implants from posterior sources were used, also show definitely that the determination of the type of bud to be formed is the result of the interaction of implant and host. In the region of segments 1–9 of hosts, implants from anterior sources produce head buds, never tails. Implants of posterior origin, however, form buds the ventral halves of which terminate in partial anal segments (24 cases). In 14 of these tail buds the dorsal portions ended in truncate regions. In each of the other 10 cases there was an undifferentiated, conical or slender part associated with the dorsal region of the end of the bud. One of these dorsal structures (at the sixth segment) was a cone with a proboscis-like part on its ventral side. This was the only case in which any of these buds included a part which might be interpreted as showing a feature characteristic of the head. On the basis of these implants inserted into anterior segments of hosts, it might be thought that there is an inhibitory effect of the host's head which prevents development of a head bud except in the presence of an implant taken from a strongly head-forming region of the donor. That such is not the case is shown by an analysis of the results when implants are inserted at more posterior levels of hosts.

The region of segments 10–12 is a transition zone in the case of each type of implant. Definitive buds arising as a result of implants of anterior origin fall into 4 groups: (1) complete heads, which are common at the tenth segment but rare at the twelfth and thirteenth segments; (2) buds terminating dorsally in partial anal segments and ventrally in structures which may be interpreted as possessing weak head features; (3) buds which terminate dorsally in partial anal segments and ventrally in undifferentiated cones; (4) buds each of which terminates dorsally in a partial anal segment and ventrally in a truncate region at the level of the base of the anal segment. This fourth type is rare at the tenth segment but the predominant type at the eleventh and twelfth segments. On the other hand, buds induced in this mid-body region by implants from posterior sources are of 2 general types: (1) complete tails and (2) buds terminating ventrally in partial anal segments but dorsally in truncate regions. One of the significant points about these buds with partial anal segments is that in buds induced by implants of anterior origin it is the dorsal side of the anal segment which is present while in buds arising from implants of posterior origin it is always the ventral part of the anal segment which develops.

At posterior levels of hosts, the anterior implants produce practically complete tail buds occasionally, but in most cases they form buds terminating dorsally in partial anal segments and ventrally in truncate regions. Not one head bud appeared at these posterior levels. In only

one bud was there an extra part in addition to the dorsal part of the anal segment. This extra part was difficult to interpret but suggested, in general appearance and behavior, a proboscis. On the other hand, implants from posterior sources inserted in this posterior region give rise to complete tail buds only.

It seems evident from these results that there is no marked inhibitory influence exerted by the host's head to prevent development of a head bud. If there were such an influence, anterior implants should be able to induce head buds more easily at these posterior levels than in the mid-body or anterior regions. Actually, however, it is in the anterior region that head buds are most readily formed.

Another general point, evident from these experiments, is that the organization of the ventral half of the terminal region of a bud is apparently determined, in most cases at least, by the implant while differentiation of the dorsal half of the bud is under the control of the host. Thus when an implant from an anterior source is inserted into an anterior segment, even as far posterior as the ninth segment, a complete head bud results from the presence of the two combining sets of head-determining factors. Similarly, when an implant from a posterior source is inserted into a posterior segment, a complete tail bud is developed. When, however, anterior implants are inserted at posterior levels, the terminal portion of each definitive bud consists of a truncate region ventrally and a partial anal segment dorsally. The host apparently determines the tail while the implant is relatively ineffective as a determining agent. On the other hand, when posterior implants are inserted at anterior levels the terminal region of each resulting definitive bud is made up of a partial anal segment ventrally and either a truncate region or a conical elevation dorsally. In this group, therefore, the implant apparently determines the tail while the host is comparatively ineffective, although making a slight contribution to the determination of the organization of the bud. In both of these cases it is the tail portion of the bud which is formed and the head-determining factor which is weak. Apparently the determining factor for tail formation present in the posterior region of a *Clymenella* is much stronger than the corresponding factor for head formation present in the anterior region, irrespective of which of these factors is in the implant and which is in the host.

Regarding the number of segments present in the buds, the results are not very definite. In the first place, more data on this point are necessary. Although 42 definitive buds resulted from these implants from posterior sources, segmentation was absent or weak in 24 of them. In 9 others it was impossible to go beyond an estimation of the possible number of segments present. In one case a bud of 7 segments was formed

at the fifteenth segment following an implant from segments 14–15 of the donor. The remaining 8 buds indicate that the implant may be more important than the host in determining the number of segments in these tail buds. Implants from 14–15 induced 2 of these buds, both of 7 segments, one developing at the tenth segment, the other at the eleventh segment. Implants from 13–14 or 14 induced 3 buds as follows: one of 7 segments which developed at the first segment of the host; one of 8 segments which was formed at the fourth host segment; one of 8 segments which developed at the sixth segment of the host. Implants from 12–13 or 13 induced the remaining 3 tail buds. These buds were: one of 8 segments at the first segment of the host, one of 10 segments at the fourth segment, and one of 9 segments at the seventh host segment. Although the number of segments in each of these buds was approximately the same as the number posterior to the source of the implant, it should be noted that in every case there were fewer segments than were present in the host posterior to the level of implantation. Since it is common for implants to induce buds which include fewer segments than occur in the corresponding region of the host, this whole matter requires additional study.

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SOME FACTORS WHICH INFLUENCE OXYGEN CONSUMPTION BY BACTERIA IN LAKE WATER [1]

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The conditions which influence the distribution of oxygen in lake water are the subject of an extensive literature (Thienemann, 1928; Grote, 1934), but there is relatively little information on the rôle played by bacteria regardless of the alleged importance of these organisms as agencies which deplete the oxygen content of natural waters. Birge and Juday (1911) state that the respiration of the bacteria of decay is more important than the respiration of aquatic animals in the utilization of oxygen. According to Domogalla, Fred and Peterson (1926) the oxygen content of lake water below the photosynthetic zone is inversely proportional to the abundance of bacteria. Similarly, Kusnetzow and Karsinkin (1931) report that the zones of diminished oxygen tension in Lake Glubokoje are caused primarily by bacterial activity. Seiwell and Seiwell (1938) attribute the oxygen minimum layer in the sea to the increased decomposition of organic matter by bacteria in such zones. Pütter (1924) expresses the view that bacteria consume more oxygen in sea water than all other organisms combined.

Some workers have assumed erroneously that the amount of oxygen consumed by bacteria in water can be evaluated merely by storing samples of raw water in the dark in glass-stoppered bottles and determining the oxygen content after different periods of incubation at the *in situ* temperature. This procedure may serve to estimate the amount of materials in such water which can be oxidized by bacteria but it fails to indicate the rate or the amount of oxygen consumption *in situ* because the storage of water in the laboratory is always accompanied by increased and altered bacterial activity (ZoBell and Anderson, 1936). Therefore, in order to ascertain how much oxygen is consumed by bacteria in lakes, several factors which influence oxygen consumption must be considered.

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[2] Visiting investigator on sabbatical leave from the Scripps Institution of Oceanography, University of California, La Jolla.

Influence of Temperature

Water temperatures ranging from 0° to 35° C. or higher occur in lakes. In order to evaluate the effect of temperature upon the rate at which bacteria consume oxygen, the following experiments were conducted. Water was collected from Lake Mendota in 5-gallon carboys and thoroughly shaken to insure uniformity of its bacterial and chemical composition. It was then siphoned into each of several dozen 145 ml. oxygen bottles. The latter were divided into four lots and transferred to water baths held at 8°, 18°, 25° and 37° C. respectively. Duplicate bottles from each lot were analyzed for the oxygen content of the water by the Winkler method at the beginning of the experiment and after different periods of incubation. In most cases duplicate determinations agreed to within 0.03 mgm./1. and additional bottles were analyzed if the divergence of duplicates exceeded 0.10 mgm./1. The bottles were incubated in the dark to prevent photosynthetic activity. Table I shows the amount of oxygen which was consumed by the bacteria after different periods of incubation.

TABLE I

Amount of oxygen consumed by bacteria in Lake Mendota water incubated at different temperatures. The water initially contained 9000 bacteria per ml. and 7.46 mgm. of oxygen per liter.

Incubation temperature	Milligrams of oxygen consumed per liter after				
	2 days	5 days	10 days	20 days	30 days
8° ± 0.3° C.	0.09	0.26	0.61	1.32	2.84
18° ± 0.3° C.	0.31	1.43	2.41	3.06	3.77
25° ± 0.1° C.	0.56	2.02	2.37	3.95	4.49
37° ± 0.2° C.	0.49	1.84	2.95	4.11	4.76

A multiplicity of correlative factors are involved in the interpretation of the results. In the first place it must be recognized that the rate of reproduction and the death rate of bacteria as well as their respiratory rate are influenced by the temperature of the water. During the first 24 hours the bacterial population of the water incubated at 8° C. had trebled while that incubated at 25° C. had increased nearly a hundredfold. The bacterial population of the water incubated at 25° C. reached its maximum on the third day after which it decreased. The bacterial population of the water incubated at 18° C. reached its maximum on the fourth day and then decreased. The bacterial population of the water incubated at 8° C. was still increasing after ten days at which time

it contained more bacteria than the water incubated at the higher temperatures.

The residual organic matter becomes more and more refractory to oxidation as the more oxidizable fractions are oxidized and finally a lack of oxidizable matter limits further oxygen consumption. Although six times as much oxygen was consumed by multiplying cultures in two days at 25° C. as at 8° C., there was virtually no difference after sixty days because at all temperatures most of the readily oxidizable organic matter had been oxidized. By applying the formula of Buchanan and Fulmer (1930):

$$m = \frac{2.303\ S \log b/B}{t(b - B)}$$

where m is the oxygen consumed per cell in time t; S the total amount of oxygen consumed in time t; B the number of bacteria at the beginning of the experiment and b the number after time t; it was found that during the first 24-hour period of incubation at 25° C. the bacteria in Lake Mendota water consumed 21 to 43×10^{-12} mgm. of oxygen per cell per hour. Although the bacterial population continued to increase throughout the second 24-hour period, the oxidizable organic matter content of the water was diminished to such an extent that the bacteria used only 5.4×10^{-12} mgm. of oxygen per cell per hour. After 5 days the bacteria incubated in water at 25° C. were using only 0.5×10^{-12} mgm. of oxygen per cell per hour. During the first 24-hour period of incubation at 8°, 18°, 25° and 37° C., the bacteria consumed an average of 9×10^{-12}, 20×10^{-12}, 32×10^{-12} and 61×10^{-12} mgm. of oxygen per cell per hour respectively. Bacteria from Lake Glubokoje were found by Liagina and Kusnetzow (1937) to consume an average of 18.8×10^{-12} mgm. of oxygen per cell per hour at 10° C. and 30.3×10^{-12} mgm. at 15° C.

The influence of temperature upon the rate of bacterial respiration independent of its effect on bacterial multiplication and other variables mentioned above was investigated by inoculating lake water enriched with 0.01 per cent each of glucose and asparagine with about a hundred million bacteria per ml. The inoculant was prepared by washing the actively multiplying colonies from several bottles of nutrient agar previously inoculated with the mixed microflora from Lake Mendota and incubated at 18° C. Under these conditions the rate of oxygen consumption was found to increase exponentially with the temperature with one exception as shown by Fig. 1. The Q_{10} from 8° to 25° C. was found to be 2.1. The decelerating rate of respiration in the water incubated at 37° C. is attributed to the inactivation of some of the

thermo-sensitive bacteria or their enzymes and is not due to a lack of either oxidant or oxygen. According to Edwards and Rettger (1937) the respiratory enzymes of bacteria are especially thermo-sensitive, many being destroyed at relatively low temperatures. Similarly ZoBell and Conn (1940) have shown that prolonged exposure to temperatures exceeding 25° C. is injurious to many water bacteria and that the respiratory enzymes of some are destroyed in 10 minutes at 30° C.

Influence of Oxygen Tension

According to Amberson (1928) the rate of oxygen consumption by unicellular organisms is independent of the oxygen tension over a wide

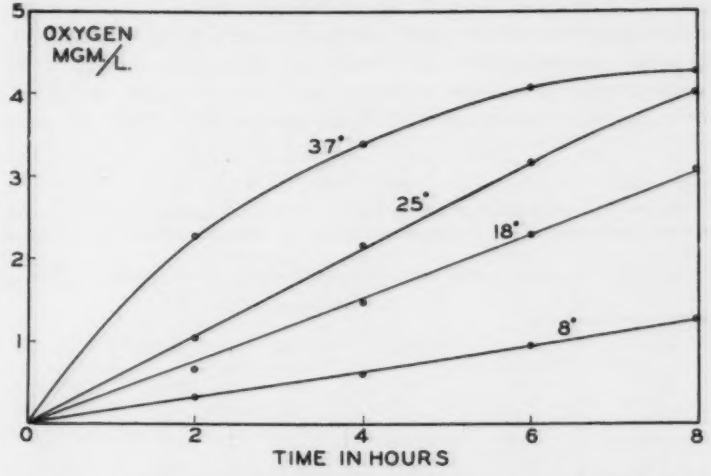

FIG. 1. Oxygen consumed after different periods of time at 8°, 18°, 25° and 37° C. by lake bacteria (resting cells) in lake water enriched with 0.01 per cent each of glucose and asparagine.

range. Similarly Pütter (1924), Harvey (1928), Pomeroy (1938) and others report that the rate of oxygen uptake by bacteria is not influenced by the oxygen tension. However, Waksman and Carey (1935), Heukelekian (1936), Schlayer (1936) and others find that the rate of oxygen consumption by certain bacteria is proportional to the oxygen tension. These antithetical observations may be due to variations in experimental procedure.. For example, Kempner (1937) finds that at relatively low temperatures or with old cells the respiration of *Escherichia coli* is independent of the oxygen tension, but when using

heavy suspensions of young cells at their optimum temperature, the oxygen uptake of *E. coli* is a function of the oxygen tension. These observations suggest that all of the oxygen may be consumed in the immediate vicinity of groups or clumps of young cells more rapidly than it can reach them by diffusion. Clifton and Logan (1939) and others have shown that young cells consume oxygen much faster than old ones. According to Waksman and Renn (1936) the effect of oxygen tension is most pronounced when resistant materials are being oxidized.

The following experiments were performed to ascertain what effect the oxygen tension has upon the respiration of bacteria in lake water. The oxygen content of water recently collected from Lake Mendota was adjusted by bubbling either nitrogen or oxygen through it until otherwise identical lots of the water contained from 0.31 to 8.26 mgm./l. This was siphoned into oxygen bottles and the oxygen tension of the water was determined after different periods of incubation at 25° C. The results of the experiment, which are summarized in Table II, show

TABLE II

Oxygen consumed by multiplying bacteria in Lake Mendota water containing different concentrations of dissolved oxygen after different periods of incubation at 25° C.

Initial dissolved oxygen	Oxygen consumed after				
	1 day	2 days	3 days	5 days	10 days
mgm./l.	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*
0.31	0.21	0.29	—	—	—
0.85	0.23	0.41	0.54	0.79	—
1.78	0.28	0.46	0.61	0.95	1.39
4.02	0.27	0.39	0.50	0.92	1.32
8.26	0.28	0.42	0.56	1.06	1.52

that within the limits of experimental error the oxygen tension of the water does not influence the rate at which oxygen is consumed by respiring bacteria.

In several other experiments which have been reported in detail by ZoBell and Stadler (1940*a*), it was similarly found that the respiration of "resting" cells in enriched lake water as well as cultures of bacteria is independent of the oxygen tension over a wide range. Although there is a slight tendency for the oxygen tension of lake water to influence the multiplication of bacteria, there is no evidence to indicate that the oxygen uptake of bacteria is influenced by the oxygen tension until

the oxygen content is as low as 0.3 mgm./1. Even when the water was supersaturated with oxygen (as much as 26.48 mgm./1.), bacteria consumed oxygen no more rapidly than when the water contained only 0.3 mgm./1. As the oxygen tension is reduced below 0.3 mgm./1. the rate of bacterial respiration decreases rapidly.

Influence of Organic Matter

According to Friedlein (1928) 100 mgm. of utilizable organic matter per liter approximates the minimum concentration in which certain bacteria can grow. This is greatly in excess of the concentration of total organic matter commonly found in lakes; Lake Mendota water containing an average of only 12 mgm./1. (Birge and Juday, 1934); and, as will be shown below, much of this is highly refractory to bacterial decomposition. While many bacteria indigenous to natural waters can

TABLE III

Effect of the concentration of glucose upon the rate of respiration of "resting" bacteria in water which initially contained 7.28 mgm. of oxygen per liter.

Concentration of glucose	Oxygen consumed after			
	1 hour	2 hours	4 hours	8 hours
mgm./l.	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*
0	0.06	0.10	0.14	0.23
2	0.12	0.19	0.38	0.53
5	0.16	0.26	0.52	0.91
10	0.17	0.34	0.71	1.18
100	0.29	0.63	1.43	1.69
1000	0.32	0.69	1.38	1.75

maintain themselves indefinitely when sub-cultured in lake water as well as in sea water which contains less than 10 mgm. of total organic matter per liter, this concentration seems to be in the neighborhood of their threshold for multiplication.

The effect of concentration of organic matter upon the rate of oxygen consumption was investigated by adding from 2 to 1000 mgm. of glucose per liter of synthetic lake water inoculated with enough washed bacterial cells (mixed microflora from Lake Mendota) to give around fifty million per ml. After different periods of incubation in oxygen bottles at 25° C. the amount of oxygen consumed was determined. The results are summarized in Table III. Although the "resting" cells were washed twice by centrifuging and re-suspending in synthetic lake water to free them of oxidizable materials, they consumed about 1.0×10^{-12} mgm. of oxygen per cell per hour without the addition of any

organic matter. However, the rate of respiration was increased by the addition of glucose until a concentration of 100 to 1000 mgm./1. was reached. Similar results were obtained with glycerol, asparagine and lactic acid. The rate of respiration was not influenced by the addition of 2 mgm./1. of either ammonium sulphate or potassium nitrate thereby proving that the lack of available nitrogen was not a limiting factor. Further experiments are planned to determine the effect of concentration of other organic compounds upon the respiration and other vital activities of bacteria.

TABLE IV

Relative rates of oxidation of 2.0 mgm./l. of organic compounds by cultures of lake bacteria as indicated by the amount of oxygen consumed after different periods of incubation at 25° C. The "oxygen demand" is the amount of oxygen required for the complete (100 per cent) oxidation of 2.0 mgm. of the compound.

Compound	Oxygen demand	Milligrams of oxygen consumed and per cent of each compound oxidized after					
		5 days		10 days		20 days	
	mgm.	*mgm.*	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*
Succinic acid	0.88	0.42	47.8	0.63	71.6	0.83	94.4
Glycine	1.28	0.74	57.8	0.96	75.0	1.16	90.7
Asparagine	1.46	0.59	40.4	0.95	65.1	1.24	85.0
Lactic acid	2.12	1.29	60.9	1.76	83.0	2.10	99.1
Glucose	2.14	1.56	72.9	2.02	94.5	2.07	96.8
Starch	2.36	0.44	18.7	1.19	50.4	2.20	93.3
Cellulose	2.36	0.17	7.2	0.42	17.8	0.96	40.7
Glycerol	2.44	1.63	66.8	1.97	80.8	2.36	97.8
Propionic acid	3.04	0.72	23.7	1.38	45.4	2.24	73.7
Butyric acid	3.64	0.68	18.7	1.49	40.9	2.35	64.6
Lignin	3.70	0	0	0	0	0.23	6.2
Ethanol	4.16	2.06	49.6	3.12	75.0	4.01	96.4

The susceptibility to bacterial attack of different organic compounds which may occur in lacustrine materials was tested by adding 2.0 mgm./1. of the compounds to synthetic lake water (a mineral solution simulating lake water in composition but devoid of oxidizable substrates). The latter was inoculated with mixed microflora from Lake Mendota and distributed in 145 ml. glass-stoppered bottles. In the course of the experiment the bacterial population increased from an initial 26,000 per ml. to several million per ml. after 5 to 20 days incubation at 25° C. Dissolved oxygen was determined at the beginning of the experiment and after different periods of incubation. Some of the data are summarized in Table IV which shows the rapidity with which various com-

pounds are attacked by lake bacteria. Corrections have been made for the 0.07, 0.12 and 0.16 mgm./1. of oxygen which was consumed after 5, 10 and 20 days respectively in the inoculated controls to which no organic matter was added. The "oxygen demand" is the theoretical amount of oxygen required for the complete oxidation of 2.0 mgm. of the compound to carbon dioxide and water. The oxygen demand of the nitrogenous compounds would be somewhat higher if the ammonium resulting from their decomposition were oxidized to nitrite or nitrate.

From Table IV it will be observed that there is considerable difference in the amount of oxygen required for the complete oxidation of similar quantities of different compounds and that there is even a greater difference in the susceptibility of the compounds to bacterial decomposition. Part of the difference in the rate at which oxygen is consumed by bacteria in the presence of different compounds is attributable to differences in the number of bacteria in the mixed culture which are capable of attacking the given compound as well as to differences in the growth-promoting properties of the compounds. Moreover, the fact that the oxidation of some compounds requires four or five times as much oxygen as equal concentrations of other compounds might influence the results. An evaluation of these and other factors which are involved in the full interpretation of the experiment awaits further investigations but the experiment does indicate in a general way how small concentrations of certain organic compounds might influence oxygen consumption by bacteria in lake water. It seems especially noteworthy that 2.0 mgm./1. of some of the compounds are almost quantitively oxidized in 10 to 20 days and the more resistant compounds including cellulose and lignin are slowly oxidized by certain lake bacteria. Working with eleven samples of purified lignin differing either in source or method of preparation ZoBell and Stadler (1940*b*) found that 4.4 to 14.7 per cent of the lignin was oxidized by lake bacteria in 30 days at 28° C. Kinkel (1936) has reported on the decomposition of cellulose, chitin and pectin by aerobic bacteria found in Wisconsin lakes.

Lacustrine Organic Matter

Most of the organic matter which occurs in lake water is quite refractory to bacterial oxidation. This was demonstrated by noting the rate and amount of oxygen consumption in samples collected from various Wisconsin lakes at different times during the year. The water was collected in 5-gallon carboys, filtered through cotton gauze to remove gross particles, thoroughly shaken to insure uniformity in composition and after it was warmed to 25° C., the water was siphoned into 145 ml.

oxygen bottles. Duplicate bottles were analyzed for dissolved oxygen after different periods of incubation in the dark in a water bath at 25° C. Table V shows the oxygen-consuming capacity of Lake Mendota water collected near the surface at Station S 7 from October 11, 1938, to May 6, 1939. In February the samples were collected through twelve to twenty inches of ice.

Assuming the total organic matter content of Lake Mendota water to be 12 mgm./l., the average found by Birge and Juday (1926) over

TABLE V

Biochemical oxygen demand (B.O.D.) of Lake Mendota water from Station S 7 after different periods of incubation at 25° C. Upon equilibration with air at 25° C. the oxygen concentration of the water was around 8.5 mgm./l. at the beginning of each experiment.

Description of samples			Oxygen consumed after				Ratio 5:20 day B.O.D.
Date of collection	Oxygen content	Temperature of water	5 days	10 days	20 days	30 days	
	mgm./l.	*° C.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*per cent*
10/11/38	8.96	16.4	2.06	—	3.84	5.14	53.6
10/25/38	9.08	15.1	2.30	3.82	4.51	5.58	50.9
11/ 2/38	9.37	12.8	2.17	3.73	4.17	—	52.1
11/ 9/38	10.62	8.5	1.54	2.22	3.32	3.96	46.4
11/21/38	11.54	6.6	1.60	2.40	3.41	4.09	46.8
11/30/38	11.93	5.2	0.97	2.07	2.42	3.15	40.2
12/ 8/38	12.45	3.4	0.86	1.65	2.00	2.23	43.0
12/16/38	13.86	1.6	0.96	—	2.14	—	44.4
1/ 4/39	13.08	0	1.36	2.42	3.02	4.21	45.1
1/11/39	14.34	0	1.50	2.31	3.31	4.18	45.4
1/23/39	13.72	0	1.29	—	3.22	3.86	40.2
2/ 6/39	12.90	0	0.91	1.37	2.30	2.60	39.4
2/20/39	12.74	0	0.84	1.21	1.92	—	43.8
3/ 7/39	12.31	0	1.24	1.95	2.99	3.99	41.5
3/23/39	14.18	0.3	3.11	4.66	6.20	7.13	50.2
4/10/39	11.80	7.4	3.56	5.22	7.25	—	49.1
5/ 6/39	10.37	11.6	3.92	5.38	7.46	—	52.5

a period of years, and since the complete oxidation of 1.0 mgm. of lacustrine organic matter requires an average of 1.2 mgm. of oxygen, it is estimated that only 21 to 30 per cent of the total organic matter is oxidized in 20 days, and 31 to 37 per cent in 30 days at 25° C. The prolonged incubation of a few samples showed that oxygen continued to be used at a decreasing rate, but after 94 days less than 50 per cent of the organic matter was oxidized. Using the evolution of carbon dioxide as the criterion of decomposition, Allgeier, Peterson and Juday (1934) found that from 18 to 42 per cent of the organic carbon in lake water

was oxidized in 31 days at 25° C. According to Waksman and Renn (1936), about 50 per cent of the organic matter in sea water is decomposed by bacteria under similar conditions; and of this decomposed organic matter, about 60 per cent is completely oxidized and about 40 per cent is converted into bacterial cell substance.

Upon the addition of 10 mgm./1. of glucose to the lake water all of the oxygen (about 8.5 mgm./1. at 25° C.) was rapidly depleted from the lake water thereby indicating that available nitrogen is not a factor which limits the oxidation of the organic matter in lake water. Moreover, neither the rate nor the amount of oxygen consumption was influenced by the addition of 2 mgm./1. of ammonium sulphate.

The respirable or oxidizable organic content of Lake Mendota water as indicated by its oxygen-consuming capacity was found to decrease

TABLE VI

Oxygen consumed by bacteria (B.O.D.) in samples of water from Northeastern Wisconsin lakes after different periods of incubation at 25° C.

Lake	Date of collection	Oxygen consumed after			Ratio of 5:20 day B.O.D.
		5 days	10 days	20 days	
		mgm./l.	*mgm./l.*	*mgm./l.*	*per cent*
Crystal Lake	1/12/39	0.52	1.34	1.86	28.7
Crystal Lake	2/25/39	0.44	0.95	1.57	28.1
Trout Lake	2/23/39	2.27	3.41	5.33	42.6
Silver Lake	3/29/39	0.83	1.55	2.07	40.2
Little Star Lake	3/29/39	2.12	3.31	5.04	42.1

from the time of the fall overturn in October until the ice started to melt late in March (see Table V). The melting of the ice was accompanied by much terrigenous contamination which might account in part for the sudden increase of oxidizable material at this time although other factors, including an increase of phytoplankton, are involved. According to Birge and Juday (1934) the organic matter content of Lake Mendota varies very little throughout the year, centrifuge plankton constituting less than 10 per cent of the total. However, the plankton forms appear to be decomposed much more rapidly and more completely than the resistant residual dissolved organic matter in lake water.

From 65 to 75 per cent of the organic matter in the net plankton concentrate added to synthetic lake water was found to be oxidized in 20 days at 25° C. Filtering Lake Mendota water collected during the spring months through No. 25 bolting silk which removes the larger plankton forms reduced its oxygen-consuming capacity 4 to 13 per cent.

The Foerst supercentrifuge (Juday, 1926) removed enough particulate organic matter to reduce the oxygen-consuming capacity of lake water 9 to 21 per cent.

The lower the concentration of oxidizable material in lake water the more refractory it is to bacterial attack. For example, from the last of November (11/30/38) to the first of March (3/7/39) when the 20-day B.O.D. of Lake Mendota water was lowest, the ratio of the 5-day B.O.D. to the 20-day B.O.D. was also lowest; or proportionately less oxygen was consumed in 5 days when the total oxidizable content was lowest (see Table V). Similar observations were made on water samples from other Wisconsin lakes as shown in Table VI. Less oxidizable organic matter was found and the ratio of the 5-day to the 20-day B.O.D. was lower in oligotrophic Crystal Lake than in eutrophic Trout, Little Star and Silver Lakes.

TABLE VII

Oxygen consumed per 100 mgm. (dry basis) of Lake Mendota mud dredged from Station 1 in January 1939 after different periods of incubation at 25° C.

Sample No.	Mgm. oxygen consumed after				Ratio of 5:20 day B.O.D.
	5 days	10 days	20 days	30 days	
1380	1.44	2.93	3.67	4.26	39.2
1390	1.25	3.03	4.34	4.63	28.8
1406	1.73	2.78	4.01	4.56	43.1
1409	1.09	1.97	3.58	3.96	30.2
Average	1.38	2.68	3.90	4.35	35.4

The amount of biochemically oxidizable material in Lake Mendota mud was estimated by diluting dredged samples a thousandfold or more with synthetic lake water. The diluted material was equilibrated with air to satisfy any chemical oxygen deficit (Miyadi, 1934), transferred to oxygen bottles with continued shaking to insure uniformity of composition and incubated in the water bath at 25° C. Duplicate bottles were analyzed for oxygen after different periods of incubation. Table VII summarizes the results. The relative slowness with which oxygen is consumed (5:20-day B.O.D. equals 35.4 per cent) shows that the material in the mud is more resistant to bacterial attack than that in Lake Mendota water. Similarly Waksman and Hotchkiss (1938) found that the organic matter in marine bottom deposits is oxidized less readily by bacteria than that in sea water.

According to Black (1929) mud from Station 1 in Lake Mendota contains an average of 13 per cent organic matter (dry basis). As-

suming that 1.2 mgm. of oxygen is required for the complete oxidation of 1.0 mgm. of the organic matter, it is calculated that 24 per cent of the organic matter in the lake deposits is oxidized aerobically in 20 days and around 29 per cent is oxidized in 30 days at 25° C. Decomposition continues slowly thereafter at a constantly decreasing rate. The organic matter in the mud is decomposed much more readily in the presence of oxygen than anaerobically because Allgeier et al. (1932) found that only 1 per cent of the total organic carbon was destroyed in 7 months under anaerobic conditions. Steiner and Meloche (1935) have shown that from 30 to 48 per cent of the organic matter in lake deposits consists of ligneous materials which are very resistant.

Little relationship was noted between the oxidizability of the lacustrine materials by permanganate (Wereščagin et al., 1931) and its oxidizability as indicated by oxygen consumption by bacteria. The latter is believed to be much more representative of the capacity of the organic matter to utilize oxygen *in situ*.

Discussion

If the heterotrophic bacteria in Lake Mendota consume oxygen at the rate of 9 to 20×10^{-12} mgm. per cell per hour (values found in the laboratory at 8° and 18° C. respectively), a hundred thousand bacteria per ml. would consume 4 to 9 mgm. of oxygen per liter of water during the six summer months when the temperature of the water ranges from 8° to 18° C. At this rate the activities of heterotrophic bacteria could account for the depletion of the oxygen from stagnant waters particularly in the lake bottom where bacterial populations exceeding a million per ml. have been demonstrated (Henrici and McCoy, 1938). While these estimates based upon laboratory observations are highly speculative, they substantiate the conclusions of field workers that bacteria probably play a very important rôle in the utilization of oxygen in lakes. When more complete data become available it should be possible to evaluate with greater precision the rôle of bacteria. Combining field and laboratory observations Liagina and Kusnetzow (1937) have calculated that "the decrease of the oxygen content of the waters of Lake Glubokoje can be covered with excess by the respiration of the water bacteria."

Although little is known concerning the abundance and activity of chemosynthetic autotrophic bacteria in lakes, those which oxidize ammonium, nitrite, hydrogen sulphide and methane may utilize appreciable quantities of oxygen. The possible significance of such autotrophs is apparent from the fact that the oxidation of 1.0 mgm. of ammonium to nitrate requires 5.15 mgm. of oxygen, the oxidation of 1.0 mgm. of

hydrogen sulphide to sulphate requires 4.23 mgm. of oxygen and the oxidation of 1.0 mgm. of methane to carbon dioxide and water requires 4.0 mgm. of oxygen. Hydrographic data (Welch, 1935) indicate the presence of from a trace to a few milligrams per liter of ammonium, hydrogen sulphide and methane in lake water from various places and there is evidence that these substances are oxidized. Kusnetzow (1934) believes that methane-oxidizing bacteria consume more oxygen in certain lakes than all other bacteria and animals combined. According to Ravich-Sherbo (1930) there is an extensive layer of sulphur bacteria in the Black Sea where they oxidize the hydrogen sulphide from the hypolimnion at the expense of the oxygen from the epilimnion. A similar localization of sulphur bacteria in the marginal zone of Lake Ritom has been reported by Düggeli (1924).

Summary

The rate of respiration of lake bacteria increases with temperature, the Q_{10} from 8° to 25° C. being 2.1. Mixed microflora from Lake Mendota were found to consume from 21 to 43×10^{-12} mgm. of oxygen per cell per hour at 25° C. Prolonged exposure at 37° C. is injurious to some of the bacteria.

The rate of respiration is independent of the oxygen concentration of the water within the examined range of 0.31 to 26.48 mgm./l. As the oxygen tension is reduced below 0.3 mgm./l. the rate of bacterial respiration decreases rapidly.

Both the character and the concentration of organic matter influence the rate of oxygen consumption by lake bacteria. The rate of respiration of " resting " cells increases as the concentration of glucose, asparagine and lactic acid is increased up to 100 to 1000 mgm./l.

About one-third of the organic matter in Lake Mendota water is readily oxidizable, the remainder being quite resistant to bacterial attack. The organic content of bottom deposits is less oxidizable than that of water, and the remains of plankton organisms are more readily oxidized by bacteria than the dissolved organic matter occurring in lake water.

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THE MODIFIABILITY OF THE DIURNAL PIGMENTARY RHYTHM IN ISOPODS

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It has been reported by several observers, among them Kleinholz (1937), that the diurnal variation in the pigment cell dispersion of isopods persists for some days after the animals have been kept in continuous darkness under laboratory conditions. Many other examples of the persistence of diurnal rhythms of one kind or another are given in the reviews of Jores (1935) and of Welsh (1938), the latter also describing some unsuccessful attempts to replace the 24-hour rhythm of animals by a 16-hour one through subjecting them to the influence of "artificial days" (8 hours of light and 8 of darkness). As recently reported (1939), our own experiments on the modification of the diurnal body temperature cycle in man by following a 21- or a 28-hour regime of living were quite successful in some individuals and not in others, indicating a strong individual variation in susceptibility to such modification. Realizing that greater numbers of individuals had to be studied in order to bring out decided group tendencies, it was determined to perform an experiment on animals, and the Bermudan isopod, *Ligia baudiniana,* already studied by Kleinholz, was chosen for the test.

These isopods can be seen in great numbers on the rocky shores near the Bermuda Biological Station and are easily captured. The problem of keeping them alive for more than 2–3 days under laboratory conditions was solved, after some trials, by placing them in individual 150-cc. wide-mouthed bottles, containing a small amount of sea-water and tilted slightly, so that the isopod could remain on either the dry or the wet portion of the bottom of the bottle. A small amount of food in the form of vegetative matter scraped off the wet rocks where the isopods abounded was put into the bottles and replenished as needed. Pieces of cheesecloth were stretched across the mouths of the bottles, permitting air to circulate but preventing the escape of the isopods. Under these conditions some animals remained alive and active for one to two weeks, others for much longer periods.

Only those specimens that were quite dark when captured in the daytime and that showed themselves to be very light during the night following were retained for further observation. They were usually

divided at random into two groups, which were placed in separate darkrooms. In one of these rooms darkness prevailed all the time, while in the other light and darkness were alternated automatically by a time clock on an 18-hour cycle of 10 hours of light and 8 hours of darkness. The animals were always observed under uniform conditions of illumination, and a simple system of recording the degree of pigment dispersion was adopted: isopods were classed as definitely dark (score of 2), intermediate (score of 1) or distinctly light (score of 0). The observations were dictated to an assistant, and duplicate observations usually yielded the same scores. The group scores were obtained by adding the individual ones. Thus, a group of 20 isopods might have a pigmentation score as high as 40 and as low as zero. Observations were made 4–6 times per 24 hours, fairly evenly distributed.

The composite data obtained on six groups of isopods, from 16 to 30 to a group, are plotted in curve *A*. It will be seen that after a flattening out of the 24-hour pigmentary activity curve the animals subjected to an 18-hour cycle of light and darkness swung into a distinctly 18-hour pigmentary rhythm. The six separate group curves upon which *A* was based were practically superimposable and were all characterized by three features: (1) an initial period of 24 to 48 hours duration in which the pigmentary rhythm was still definitely diurnal; (2) a transition period, setting in earlier in groups that were started with a period of darkness beginning at noon and later in groups that were first subjected to darkness at dusk (6 P.M.), but in all groups occurring when the repetitive 8-hour shift of darkness commenced at midnight; and (3) a period of 18-hour pigmentary rhythms which may be termed paradoxical in that the greatest pigment dispersion occurred during the artificial darkness and not during the hours of light, as it takes place in nature.

Four groups of isopods, from 12 to 18 per group, were kept in continuous darkness, and these animals preserved their 24-hour cycle of pigmentary activity, as shown in composite curve *B*. They were dark during the daytime hours and pale at night, confirming the observations of Kleinholz. One group was first maintained in continuous darkness, behaving as stated above, and then subjected to the artificial 18-hour cycle. These isopods showed the same three periods of adjustment to the new routine, as did the animals that were under the influence of the 18-hour alternation of light and darkness from the time they were captured.

One group of 20 isopods was maintained under a reversed routine of darkroom illumination (darkness in the daytime and light at night), but it preserved the normal diurnal rhythm of pigmentary activity.

That means, of course, that these isopods were dark during the periods of artificial darkness (daytime) and pale when illuminated (night), resembling both the pigmentary behavior of the animals kept under continuous darkness and the paradoxical pigmentary alternations of the animals that became adjusted to the 18-hour routine.

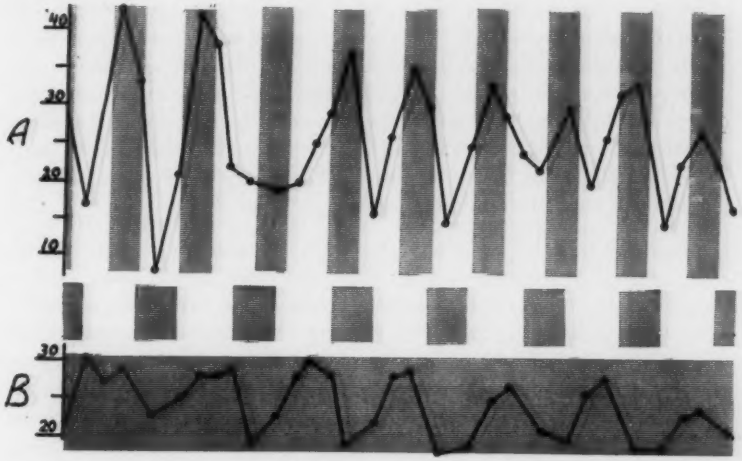

FIG. 1. Periodic variation in pigment dispersion, shown by groups of isopods, kept in the laboratory under artificial conditions of illumination and darkness. Curve *A* is based on the averages of six curves, obtained on groups comprising 30, 30, 20, 20, 16, and 20 animals, respectively. Maximal average pigment dispersion would be plotted as 46, maximum concentration, as zero. The general downward trend of the curve is due to the death of some animals, the total number decreasing from 136 to 104. The alternating areas of gray and white correspond to successive periods of 8 hours of darkness and 10 of light. Curve *B* is based on the averages of four curves, obtained on groups of 13, 12, 18, and 17 animals, respectively. Maximum pigment dispersion is plotted as 30, maximum concentration as zero. As in curve *A*, the downward trend of this curve is due to the death of individual isopods, whose total number decreased from 60 to 50. The gray background of curve *B* indicates the continuous darkness in which these groups were maintained. The alternating areas of gray and white between the two curves correspond to the natural 24-hour succession of night (7 P.M. to 5 A.M.) and day.

Although the occurrence of group pigmentary rhythms is unmistakable, the same cannot be said of individual animals. The groups studied were twice-selected isopods. It will be recalled that only those animals that were quite dark at the time of collection (daytime) were captured, in the first place. From 10 to 25 per cent of the isopods seen on the shore rocks were distinctly pale in the daytime. However, not all the animals that were dark when collected became light-colored

during the ensuing night in the laboratory. At least 10–15 per cent remained coal-black at night, and these were also rejected. Thus, the isopods retained for further observation had definitely changed from dark in the daytime to light at night. In the continued manifestation of a pigmentary rhythm the animals in captivity showed marked individual variation. Some isopods closely conformed to the group curves; others were either dark or light most of the time; still others were dark when the group was light and vice versa; finally, there were those whose pigmentary changes followed the group rhythm for one or another portion of the period of observation. As under the natural conditions of alternating day and night, so under the two artificial regimes to which the several groups of isopods were subjected, there were many individual deviations from the general group patterns of rhythmical dispersion and concentration of pigment.

Summary

Although groups of Bermudan isopods, *Ligia baudiniana,* preserve their natural diurnal pigmentary rhythm when kept in total darkness for several days, they rapidly acquire an artificial 18-hour pigmentary rhythm, if exposed to alternating periods of 10 hours of light and 8 of darkness. The new rhythm is paradoxical, pigment cell dispersion occurring during darkness, instead of during the hours of illumination.

I am greatly indebted to Dr. J. F. G. Wheeler, the Director of the Bermuda Biological Station, and to the Staff of the Station, for advice and assistance received during my stay at the Station.

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THE EFFECT OF ALKALINITY UPON MUTUAL INFLUENCES DETERMINING THE DEVELOPMENTAL AXIS IN FUCUS EGGS[1]

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Introduction

When eggs of *Fucus furcatus* develop in the dark in normal sea water in masses they tend to form rhizoids in the resultant direction of neighboring eggs (Whitaker, 1937). This phenomenon, which has been referred to as the "group effect," does not occur when only two eggs are present in a dish unless the medium is acidified. Thus, it has been reported earlier (Whitaker, 1937) that of about 200 eggs reared in pairs (at distances of 4 egg diameters or less) in sea water acidified to pH 6.0,[2] approximately 90 per cent formed rhizoids on the sides toward the neighbor, while in normal sea water at pH 7.8–8.0 only 41 per cent of about 130 eggs did so. Acidification of the medium thus strongly intensifies the "group effect."

It is of some importance in the study of the phenomenon to know whether the strong attraction which exists between two eggs in acid sea water is merely abolished at higher pH, so that each egg is unaffected by the presence of a neighbor, or whether an actual repulsion, or negative "group effect," exists. The fact that less than 50 per cent formed rhizoids toward neighbors at pH 7.8–8.0, in the count repeated above, suggests a negative "group effect," but the numbers are inadequate. Experiments have therefore been undertaken to test this point, extending the pH range as far in the basic direction as possible without much alteration of the composition of the sea water, and with special attention to exactness in pH determination.

Method

Material was obtained and treated in a manner previously described (Whitaker, 1936). Experiments were carried out during March, 1938. The eggs were kept in the dark, or in dim red light, in a constant tem-

[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

[2] Acidified either with citric acid-secondary sodium phosphate, or with HCl-$NaHCO_3$.

perature room at 15 ± ¼° C., from the time of shedding until the results were recorded. The eggs were shed and were fertilized in normal sea water, and were transferred to sea water of artificially altered pH 30–60 minutes after fertilization.

TABLE I

Conditions of the experiments. Eggs were reared two in a dish at distances of 0.3–4 egg diameters, in the dark at 15 ± ¼° C. Experiments are arbitrarily numbered in order of pH sequence. The symbol S.W. in column 2 refers to sea water. The pH difference in column 5 shows the change in pH of the medium during the experiment. The results of these experiments are presented graphically in Fig. 1.

Exp. No.	Medium				Total No. Eggs	Rhizoids toward Neighbor per cent
	Composition	pH at start	pH after 24 hrs.	pH change in 24 hrs.		
1	S.W. + KOH	8.85	8.68	−0.17	88	42
2	S.W. + Na_2CO_3 − $NaHCO_3$	8.81	8.72	−0.09	74	39
3	S.W. + KOH	8.61	8.48	−0.13	84	29
4	S.W. + Na_2CO_3 − $NaHCO_3$	8.58	8.53	−0.05	69	32
5	S.W. + Na_2CO_3 − $NaHCO_3$	8.40	8.32	−0.08	119	27
6	S.W. + Na_2CO_3 − $NaHCO_3$	8.41	8.36	−0.05	30	23
7	S.W. + KOH	8.42	8.30	−0.12	26	19
8	S.W. + Na_2CO_3 − $NaHCO_3$	8.41	8.35	−0.06	18	22
9	S.W. + Na_2CO_3 − $NaHCO_3$	8.43	8.34	−0.09	36	22
10	S.W. + Na_2CO_3 − $NaHCO_3$	8.22	8.17	−0.05	81	22
11	S.W. + Na_2CO_3 − $NaHCO_3$	8.22	8.15	−0.07	68	28
12	S.W. + Na_2CO_3 − $NaHCO_3$	8.00	8.12	+0.12	94	38
13	S.W.	8.01	7.93	−0.08	21	38
14	S.W.	8.01	8.09	+0.08	14	36
15	S.W.	7.80	7.70	−0.10	87	37
16	S.W. + citric acid	7.80	7.73	−0.07	52	42
17	S.W. + citric acid	7.61	7.67	+0.06	63	48
18	S.W. + citric acid	7.59	7.65	+0.06	42	52
19	S.W. + citric acid	7.62	7.65	+0.03	38	47
20	S.W. + citric acid	7.58	7.63	+0.05	22	45
21	S.W. + citric acid	7.40	7.43	+0.03	85	56
22	S.W. + citric acid	7.41	7.37	−0.04	32	59
23	S.W. + citric acid	7.41	7.46	+0.05	22	59
24	S.W. + citric acid	7.20	7.27	+0.07	46	76
25	S.W. + citric acid	7.22	7.21	−0.01	60	68
26	S.W. + citric acid	7.21	7.25	+0.04	24	71
27	S.W. + citric acid	6.99	7.10	+0.11	22	86
28	S.W. + citric acid	7.00	7.11	+0.11	62	76
29	S.W. + citric acid	7.02	7.08	+0.06	51	80

The pH of filtered sea water, which was collected at frequent intervals, ranged from 7.8–8.0. Samples were made more basic by adding either KOH, or a mixture of Na_2CO_3 and $NaHCO_3$. Other samples

were acidified by adding citric acid. In all cases the osmotic pressure was maintained constant at the time of these additions, and the mixture was vigorously aerated with a large scintered glass nozzle to re-equilibrate with atmospheric CO_2 tension. A glass electrode was used to measure pH, and the aeration was continued until a constant and stable pH was attained. The pH range covered was from 7.0 to 8.8 at intervals of 0.2, as closely as could be approximated. In each experiment pH was measured at the beginning, and also at the end 24 hours later. These pH values, and the composition of the media, are shown in Table I. While the glass electrode was not accurate to the second decimal of pH determination, it approached this accuracy, and therefore the values are recorded to the second decimal as read.

Sea water was not alkalinized much above pH 8.8 because of precipitation of certain salts. There is slight precipitation even at pH 8.8, and at 8.6. The development is altered in the precipitation range so that the rhizoid becomes small and very narrow at the base (Whitaker and Lowrance, 1937) although whether this is due to pH or to loss of salts from the medium is not known.

Eggs were transferred individually into experimental medium in 1 cc. Syracuse dishes by means of a very small pipette. The pipette contained experimental medium which was caused to flow about the egg before it was taken up, and then less than 0.01 cc. solution was carried over with it. Two eggs were placed in each dish at a distance from each other of 0.3–4 egg diameters. The eggs are 65–90 μ in diameter. The dishes were already mounted in moist chambers on a level platform on a vibrationless concrete table.

Results and Conclusions

The results are shown graphically in Fig. 1. Each point in Fig. 1 represents the results of one experiment, and conditions of each experiment are given in Table I.

It is clear from Fig. 1 that a negative "group effect" exists above pH 7.6. This appears to reach a maximum at pH 8.4, when approximately 80 per cent of the eggs form rhizoids on the sides (halves) away from the neighbor. Above pH 8.4 this percentage declines, but since the medium is changing due to precipitation, and the development of the rhizoid is altered, it is not certain that the results above pH 8.4 are entirely comparable. For this reason the curve is dotted in Fig. 1. This evidence of negative "group effect" between 2 eggs in the higher pH range is in agreement with the response of single eggs developing in the ends of capillary tubes in their own diffusion gradients (Whitaker and Lowrance, 1937).

In Table I and Fig. 1 the results are grouped by experiments and pH, without attention to distance between eggs, within the limits 0.3–4 egg diameters. The effect of distance may be seen by dividing the results at a given pH into categories on the basis of distance between eggs.

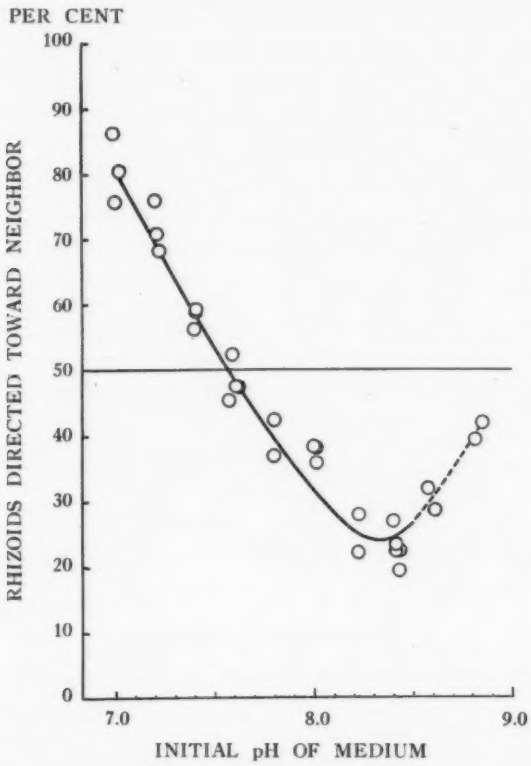

FIG. 1. Results of experiments defined in Table I, showing reversal from positive to negative "group effect" with increasing pH. Each circle represents the results of one experiment, and shows the percentage of eggs which formed rhizoids on the half toward the neighbor. The experiments are grouped at intervals of approximately 0.2 pH unit, with 129–229 eggs at each interval. The curve is dotted through pH 8.6 and 8.8 because of alteration of the medium by precipitation (see text).

At pH 7.0, the percentages of eggs forming rhizoids on the halves toward the neighbor, at different distances, are as follows: 0.3–1 egg diameter, 90 per cent; 1–2 diameters, 75 per cent; 2–3 diameters, 72 per

cent; 3–4 diameters, 70 per cent. At pH 8.4 the corresponding figures are: 0.3–1 diameter, 21 per cent; 1–2 diameters, 21 per cent; 2–3 diameters, 31 per cent; 3–4 diameters, 36 per cent. It is seen that within these limits the effect is stronger the closer the eggs, in the pH ranges of both positive and negative "group effect."

Summary

1. When two eggs of *Fucus furcatus* develop in the dark within 0.3–4 egg diameters of each other, the point of rhizoid origin and the developmental axis are influenced by the presence of the neighbor.

2. When the pH of the medium is below approximately 7.6, the rhizoids tend to form on the sides of the eggs toward the neighbor. When it is above 7.6, they tend to form on the sides away from the neighbor, most markedly at pH 8.4.

3. The effect is stronger the closer the eggs, within the limits 0.3–4 egg diameters.

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DEVELOPMENT OF HALF-EGGS OF ARBACIA PUNCTULATA OBTAINED BY CENTRIFUGING AFTER FERTILIZATION, WITH SPECIAL REFERENCE TO PARTHENOGENETIC MEROGONY

ETHEL BROWNE HARVEY

(From the Marine Biological Laboratory, Woods Hole, and the Biological Laboratory, Princeton University)

It has been shown previously (1932, 1936) that the half-eggs of *Arbacia punctulata*, which are obtained by centrifuging the *unfertilized* egg, will develop if fertilized or activated artificially. The white halves, containing the ♀ nucleus will develop often quite normally, both fertilized and parthenogenetic, into plutei; the red halves after fertilization (fertilized merogones) containing only the ♂ nucleus will develop, usually in an aberrant fashion, into plutei. The red halves will also develop parthenogenetically (parthenogenetic merogones) as far as the blastula stage, in spite of the fact that they have no nuclei whatever. Additional data with a more complete series of photographs on this subject will be published in a forthcoming number of this journal.

The present paper deals with the development of similar half-eggs of *Arbacia punctulata* which are obtained by centrifuging the *fertilized* egg. It might be expected that these halves would develop similarly to (or better than) those mentioned above, having the same nuclear (or non-nuclear) content. Such has been found, however, not to be the case; they do not develop nearly so well.

Stratification and Breaking of the Fertilized Egg

When *Arbacia* eggs which have been normally fertilized are centrifuged, they stratify into layers similar to the unfertilized egg, only the layers are not nearly so clean-cut (Photograph 1). Except for about five minutes after fertilization, the fertilization membrane prevents the elongation and breaking apart of the egg, so that it is necessary to remove this in order to obtain the half-eggs. This is removed by shaking the eggs 2 minutes after fertilization, just after the membrane has been raised and before it has hardened. The eggs were centrifuged for 6 to 8 minutes at about 10,000 × g., in an isosmotic sugar solution (approximately 2 parts sugar solution to one part sea water + eggs). At one stage of very short duration soon after fertilization, the egg tends

to break up into very small pieces when centrifuged, as noted previously (1933); at all other times, it breaks into slightly unequal halves, similar to the unfertilized egg (Photograph 2). At most stages, the white half is slightly larger than the red, but if centrifuged just before cleavage, this half tends to be considerably smaller (Photograph 3). The long streamers which have been described as characteristic of the fertilized egg at a certain stage and which are readily observed with the centrifuge microscope (1933), are preliminary to the final breaking apart and are especially noticeable with relatively low centrifugal forces (5,000 × g.). When the streamers break apart, a sort of tail is often left on the white half; this contracts after 10 to 30 minutes, leaving the white half-egg spherical. The nucleus, which soon after fertilization is the combined ♂ and ♀ nuclei, always goes to the light pole, and is therefore always in the white half, just as is the female nucleus in the unfertilized white half. The ♂ nucleus in the recently fertilized *Arbacia* egg is apparently lighter than the pigment and yolk granules and is not carried by centrifugal force into the heavy half-egg, as it is in the eggs of *Parechinus microtuberculatus, Paracentrotus lividus* and *Sphaerechinus granularis* (1934). It is only in those eggs in which the sperm has entered near the centrifugal end of the egg, as it is thrown into position in the centrifuge, and the egg centrifuged apart while the sperm nucleus is still in this position, that any nucleus is found in the red half-egg which has been completely separated off. This will be described later on. If centrifuged after the amphiaster has been formed, this also, together with the chromosomes, goes to the light pole and is segregated in the white half-egg.

Development of the White Half-egg

The early development of the white half may be fairly normal. The first cleavage divides the egg into two equal cells, through the oil cap (Photograph 4), or through the plane of stratification (Photograph 5) or diagonally; there is a delay of only a few minutes in comparison with the controls. Normal 4 and 8-cell stages follow (Photographs 6, 7), and with further cleavages (Photographs 8–10) a blastula is formed slightly later than the normally developing egg. Owing to the lack of pigment and the small size of the cells, it is difficult to be sure about the micromeres; in a few cases I have thought they were present, but usually they could not be observed. Many of the blastulae, after becoming free-swimming, develop no further though they increase slightly in size—they become "Dauerblastulae," or permanent blastulae (Photograph 11). Some of the blastulae become filled with cells and somewhat differentiated (Photograph 12), some acquire a skeleton often

in the form of a primitive triradiate spicule, sometimes more complicated, even without invagination; that is, the formation of the skeleton seems independent of the shape of the larva or other differentiation (Photograph 13). None of the blastulae has developed into a normal pluteus. The nearest approach to a pluteus is shown in Photograph 14; the skeleton approximates the normal, but there are no arms and it is only about a fifth the volume of the normal white pluteus of this age (4 days) from a white half obtained before fertilization and subsequently fertilized. Similar permanent blastulae and imperfect plutei occur together with normal plutei in the white halves fertilized after centrifugation (Photographs in succeeding paper).

Many of the white halves do not develop so normally as this. It has been noted in previous papers (1934, 1940) that the ectoplasmic layer which is formed soon after fertilization and binds the cleavage cells together is thrown off by centrifugal force. In some cases, in the

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PLATE I

Development of White Half

The photographs were all taken of living eggs and all brought to approximately the same magnification, 250 ×.

PHOTOGRAPH 1. Fertilized egg centrifuged 4 minutes after fertilization. Photographed immediately; to show stratification.

PHOTOGRAPH 2. Typical red and white halves. Centrifuged 5 minutes after fertilization. Photographed 10 minutes later. Note nucleate white halves, with contracted tail, and non-nucleate reds.

PHOTOGRAPH 3. White half much smaller than red. Centrifuged just before cleavage. Photographed one-half hour later.

PHOTOGRAPH 4. Two-cell stage, cleavage through the oil cap. Centrifuged 11 minutes after fertilization. Photographed 1 hour after fertilization.

PHOTOGRAPH 5. Two-cell stage, cleavage parallel with stratification. Centrifuged 25 minutes after fertilization, streak stage. Photographed 1 hour after fertilization.

PHOTOGRAPH 6. Four-cell stage. Centrifuged 6 minutes after fertilization, monaster stage. Photographed 1¼ hours after fertilization.

PHOTOGRAPH 7. Eight-cell stage. Centrifuged a little before cleavage. Photographed 2½ hours after fertilization.

PHOTOGRAPH 8. Eight to 16-cell stage. Centrifuged 30 minutes after fertilization, streak stage. Photographed 2½ hours after fertilization. Note red halves uncleaved.

PHOTOGRAPH 9. Early blastula. Centrifuged a little before cleavage. Photographed 7 hours after fertilization. Note red half uncleaved.

PHOTOGRAPH 10. White ciliated blastula, 1 day old. Centrifuged 40 minutes after fertilization.

PHOTOGRAPH 11. White ciliate blastula, "Dauerblastula," 2 days old. Centrifuged 15 minutes after fertilization, early streak stage.

PHOTOGRAPH 12. White blastula, 4 days old, somewhat differentiated. Same lot as above.

PHOTOGRAPH 13. Four-day white blastula with triradiate spicule. Same lot.

PHOTOGRAPH 14. Abnormal pluteus, 4 days old, same lot. Most normal pluteus found.

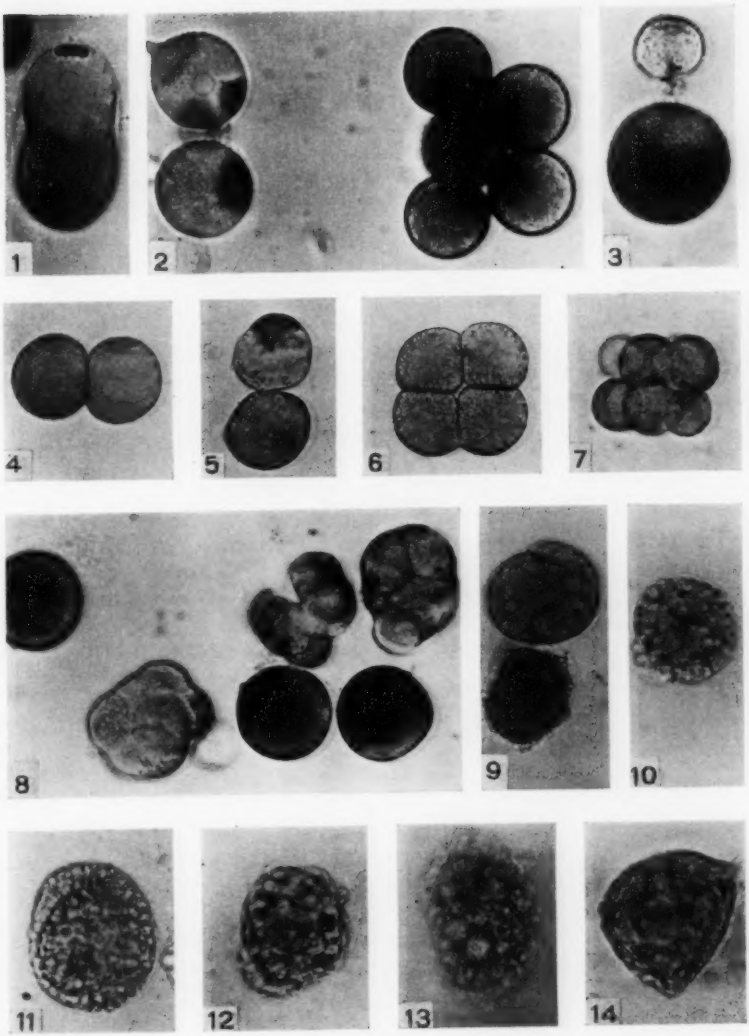

PLATE I

white halves obtained after fertilization, this layer does not regenerate sufficiently to hold the first two blastomeres together (Photograph 15). Each blastomere develops quite independently, each cleaving in regular sequence to form a white blastula (Photographs 16–21). A pair of white twins is formed which swim at first in pairs quite similar to (but of course smaller than) the twins of the whole egg obtained by centrifuging the fertilized egg at the two-cell stage (1940). These white twins gastrulated, but those isolated had formed no skeleton after 3 days.

Together with these two modes of development of the white halves a less normal development may take place. The white half after having been broken off by centrifugal force, often becomes amoeboid, or it may become amoeboid after several cleavages (Photographs 22–24). It also frequently forms a number of loosely united cells which by further cleavages give rise to a large mass of unorganized cells (Photographs 25, 26). The lack of the ectoplasmic layer is no doubt responsible for this scattering of cells.

Red Half-egg

The red half-egg contains no nucleus but consists of protoplasm which has previously been normally fertilized. These halves often do

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PLATE II

Development of White Half, Continued

PHOTOGRAPH 15. First two white blastomeres remain apart. Centrifuged just before cleavage. Photographed 1½ hours after fertilization. Note red half uncleaved.

PHOTOGRAPH 16. Each white blastomere has cleaved independently. Centrifuged 23 minutes after fertilization. Photographed 1¾ hours after fertilization.

PHOTOGRAPH 17. Each white blastomere is 4-celled. Centrifuged 6 minutes after fertilization. Photographed 2 hours after fertilization.

PHOTOGRAPH 18. Late cleavage of white twins. Centrifuged 40 minutes after fertilization. Photographed 4 hours after fertilization. Note whole blastula below.

PHOTOGRAPH 19. White twin blastulae after becoming free-swimming. Centrifuged 23 minutes after fertilization. Photographed 7 hours after fertilization.

PHOTOGRAPH 20. White twin blastulae, with undeveloped red half. Centrifuged a little before cleavage. Photographed 7½ hours after fertilization.

PHOTOGRAPH 21. A similar pair with whole blastula in comparison. Centrifuged 40 minutes after fertilization. Photographed 7½ hours after fertilization.

PHOTOGRAPH 22. White half amoeboid. Centrifuged 23 minutes after fertilization. Photographed 1½ hours after fertilization.

PHOTOGRAPH 23. White half very amoeboid. Centrifuged 42 minutes after fertilization. Photographed 1½ hours after fertilization.

PHOTOGRAPH 24. White half amoeboid after several cleavages. Centrifuged just before cleavage. Photographed 3 hours after fertilization. Red half undeveloped.

PHOTOGRAPH 25. White halves form scattered cells. Centrifuged 30 minutes after fertilization. Photographed 2½ hours after fertilization.

PHOTOGRAPH 26. Same group as one shown in Photograph 25, after further cleavages, 1 hour later.

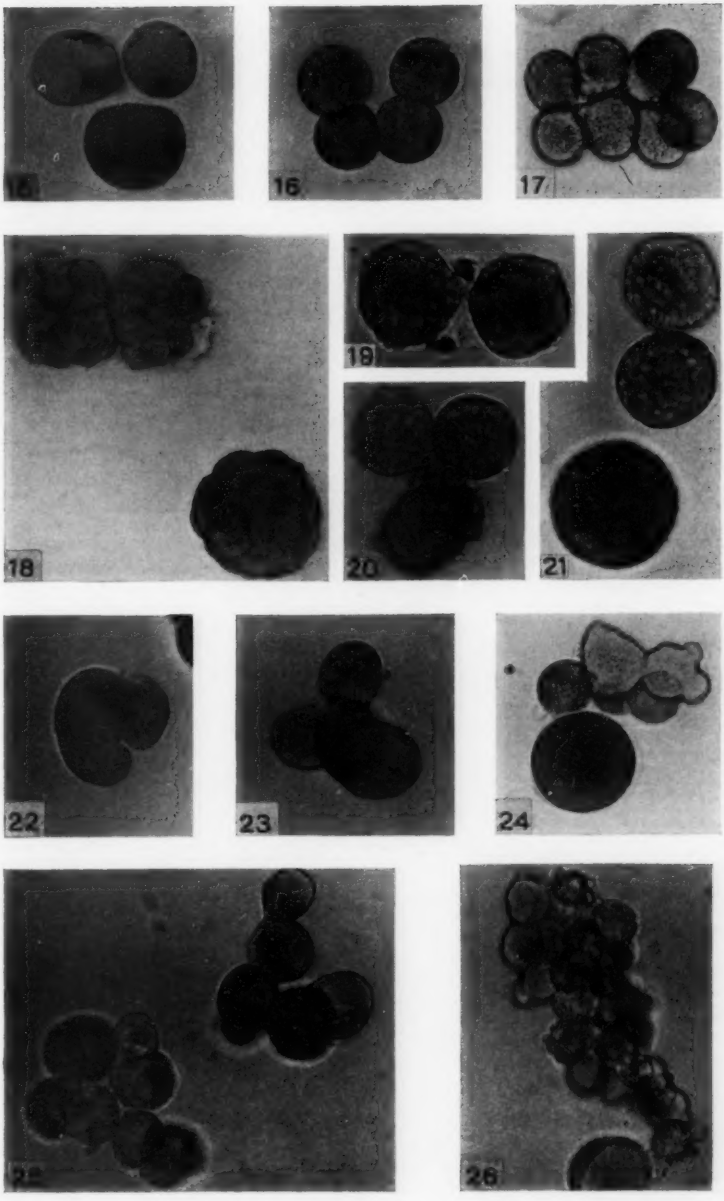

PLATE II

not change at all even after several hours and do not develop (Photograph 27). In many cases, however, they show signs of activation in that they become amoeboid (Photographs 28, 29). Often a notch appears in the middle of the egg, as though it were about to cleave (Photographs 30, 31). A series of photographs (Photographs 33–36), at intervals of about 10 minutes, indicates that the cleavage does not actually take place, but the notch seems to be an abortive attempt to cleave and soon disappears. The red half sometimes breaks up into a number of small spheres (Photograph 32), but this seems to be by a pinching-off process rather than cleavage as there are no preliminary stages except the amoeboid processes noted above, and the spheres are unstable, that is, they come and go, and they do not become progressively smaller. That the protoplasm is activated and different from unfertilized protoplasm is indicated also by the occasional appearance of a large monaster (Photographs 37, 38), and in one red half a beautiful amphiaster was observed, but this did not lead to cleavage (Photograph 39).

It makes very little difference at what stage after fertilization the eggs are broken apart by centrifugal force. It seemed to me reasonable to suppose that after the nuclear membrane had broken and liberated its contents into the cytoplasm, then this cytoplasm would be different, and the red half would be more likely to cleave than previously. This was, however, not the case. The red halves obtained after the breakdown of the nuclear membrane act exactly like those obtained while the nuclear membrane is still intact; there is no cleavage. This means either that all the achromatic material from the nucleus goes together with the chromosomes and spindle into the light half, or else that it has no effect on the protoplasm, in enabling it to cleave.

PLATE III

Red Halves

PHOTOGRAPH 27. Red halves unchanged after 5 hours. Centrifuged 15 minutes after fertilization.

PHOTOGRAPHS 28, 29. Red halves amoeboid. Centrifuged 30 minutes after fertilization, streak stage. Photographed 4 hours after fertilization.

PHOTOGRAPH 30. Red halves with notch as though ready to cleave. Centrifuged a little before cleavage. Photographed 2½ hours after fertilization.

PHOTOGRAPH 31. Red halves notched and amoeboid. Centrifuged 30 minutes after fertilization. Photographed 3 hours after fertilization.

PHOTOGRAPH 32. Red halves pinched into small fragments. Centrifuged 10 minutes after fertilization, monaster stage. Photographed 5 hours later.

PHOTOGRAPHS 33–36. One red half at intervals of 10 minutes. Centrifuged a little before cleavage. Photographed 1½–2 hours after fertilization.

PHOTOGRAPHS 37, 38. Red halves with monaster. Centrifuged 6 minutes after fertilization. Photographed 3 hours later.

PHOTOGRAPH 39. Red half with amphiaster. Centrifuged 21 minutes after fertilization. Photographed 2 hours later.

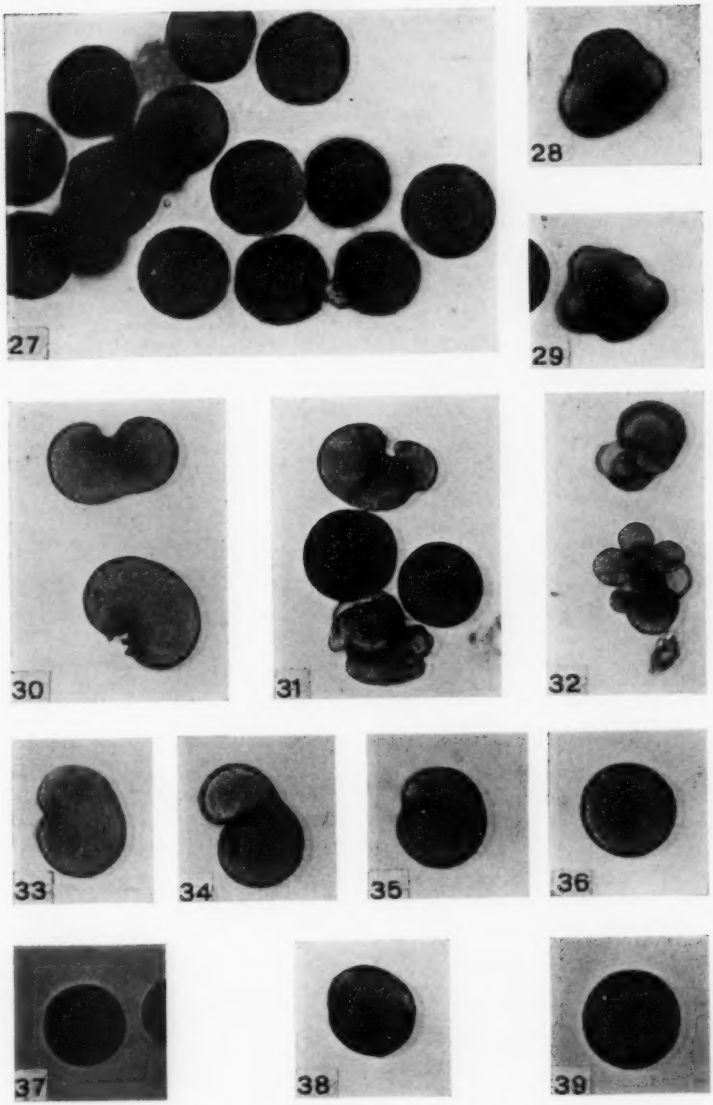

PLATE III

Re-activation of Halves

The red halves obtained by centrifuging the fertilized egg cannot be re-fertilized nor can they be artificially activated. Even if obtained as quickly as possible after fertilization, re-activation has practically no effect. This is true also of the white halves; there was only a slightly greater tendency toward amoeboid activity and disorganized masses of cells if treated with the hypertonic solution which causes parthenogenesis in the unfertilized halves.

Development of Halves Obtained Immediately After Fertilization

As mentioned above, it is only if one centrifuges the eggs immediately after fertilization, that one can obtain any cleavage in the red halves. This cleavage is due to the presence of the sperm which entered near the centrifugal pole, and the egg has broken apart before the sperm nucleus has had a chance to approach the ♀ nucleus at the centripetal pole. It is well known that the sperm may enter the *Arbacia* egg at any point on the surface, and also that the eggs fall at random in the centrifuge tubes without any orientation. A group of red halves from eggs centrifuged immediately after fertilization is shown in Photograph 40. It is seen in the next photograph (41) that one of these halves contains the ♂ nucleus, and in the two succeeding photographs (42, 43) it is shown that this half-egg cleaves whereas the others do not. If the red half contains the ♂ nucleus, it develops similarly to a

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PLATE IV

Development of Halves Obtained Immediately After Fertilization

PHOTOGRAPH 40. Group of red halves centrifuged off 3 minutes after fertilization. Photographed 1 hour later; monaster in center cell.

PHOTOGRAPH 41. Same group ½ hour later; ♂ nucleus in center cell.

PHOTOGRAPH 42. Same group ½ hour after 41; center cell cleaving. Monaster in egg above to left.

PHOTOGRAPH 43. Same group 20 minutes after 42; center cell completely cleaved, with nucleus in each cell. ♂ nucleus in egg above.

PHOTOGRAPH 44. Development of red half with ♂ nucleus; 3-celled, multinucleate. Same set as above. Photographed 6 hours after fertilization.

PHOTOGRAPH 45. Development of red half, multinucleate. Centrifuged 1 minute after fertilization. Photographed 7 hours later.

PHOTOGRAPH 46. Group of small pigmented fragments, one with ♂ nucleus. Centrifuged 1 minute after fertilization. Photographed 1 hour later.

PHOTOGRAPH 47. Same lot, another fragment 4 hours later; now 2-celled.

PHOTOGRAPH 48. White half centrifuged 4 minutes after fertilization. Photographed 1½ hours later; normal development.

PHOTOGRAPH 49. White half centrifuged 3 minutes after fertilization. Photographed 3 hours later; normal development.

PHOTOGRAPH 50. White half centrifuged 4 minutes after fertilization. Photographed 3 hours later; disconnected cells.

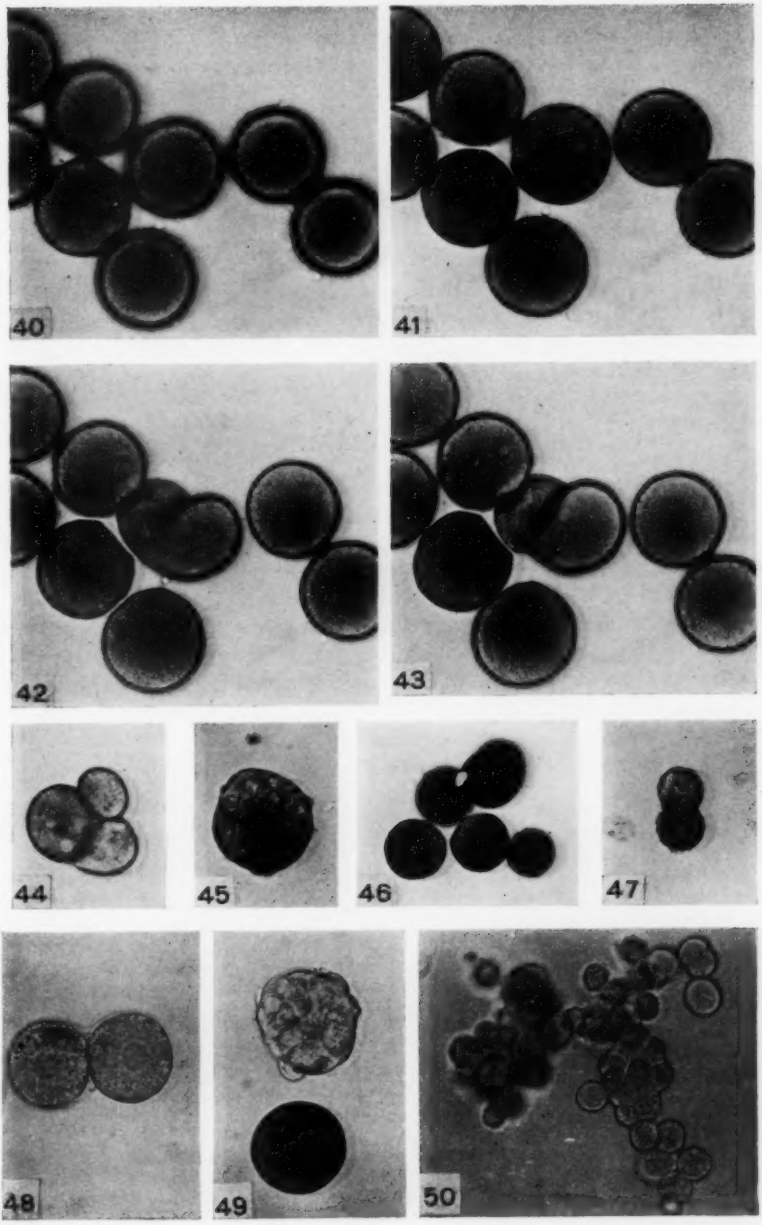

PLATE IV

red half fertilized after being separated from the white half (i.e. a fertilized merogone), nuclear divisions often taking place without cell divisions (Photographs 44, 45). If centrifuged at a certain period soon after fertilization when the eggs tend to break into small pieces, it occasionally happens that the sperm is entrapped in one of the small heavy fragments (Photograph 46, center cell). This, though quite small, will cleave (Photograph 47); similar fragments without the male nucleus do not cleave.

The white halves obtained by centrifuging the eggs immediately after fertilization, and usually containing both nuclei [1] develop similarly to those broken off at later periods, sometimes cleaving quite regularly (Photographs 48, 49), and sometimes forming irregular masses of disconnected cells (Photograph 50).

Comparison with Parthenogenetic Merogones, and Discussion

The lack of development of the red halves from the fertilized egg is in sharp contrast to the development of the red halves obtained from the unfertilized egg and subsequently activated artificially, the parthenogenetic merogones. These, as I have shown (1936), will cleave in a fairly orderly fashion until they become blastulae. Photographs 51–54 show some successive stages in the development of a group of the parthenogenetic merogones, the unfertilized red halves (51), a group soon after activation (52), and two successive stages in cleavage (53, 54). A few more photographs of fairly normal cleavages are also

PLATE V

Parthenogenetic Merogones

PHOTOGRAPH 51. Group of red halves obtained by centrifuging the unfertilized egg.

PHOTOGRAPH 52. Similar red halves 3 hours after activation with hypertonic sea water. Note fertilization membrane and ectoplasmic layer, and monaster in lower right egg.

PHOTOGRAPH 53. Similar red halves 4 hours after activation. Early cleavage.

PHOTOGRAPH 54. Same lot of eggs ½ hour later. Further cleavages.

PHOTOGRAPH 55. Four-cell parthenogenetic merogone, 4 hours after activation.

PHOTOGRAPH 56. Eight-cell stage, 4½ hours after activation.

PHOTOGRAPH 57. About 16-cell stage, 7 hours after activation.

PHOTOGRAPH 58. Early blastula of parthenogenetic merogone, 27 hours after activation.

[1] For development of these white halves as watched with the centrifuge microscope see 1933, p. 394; it is difficult to tell whether they have both ♂ and ♀ nuclei or only the ♀ in mass cultures.

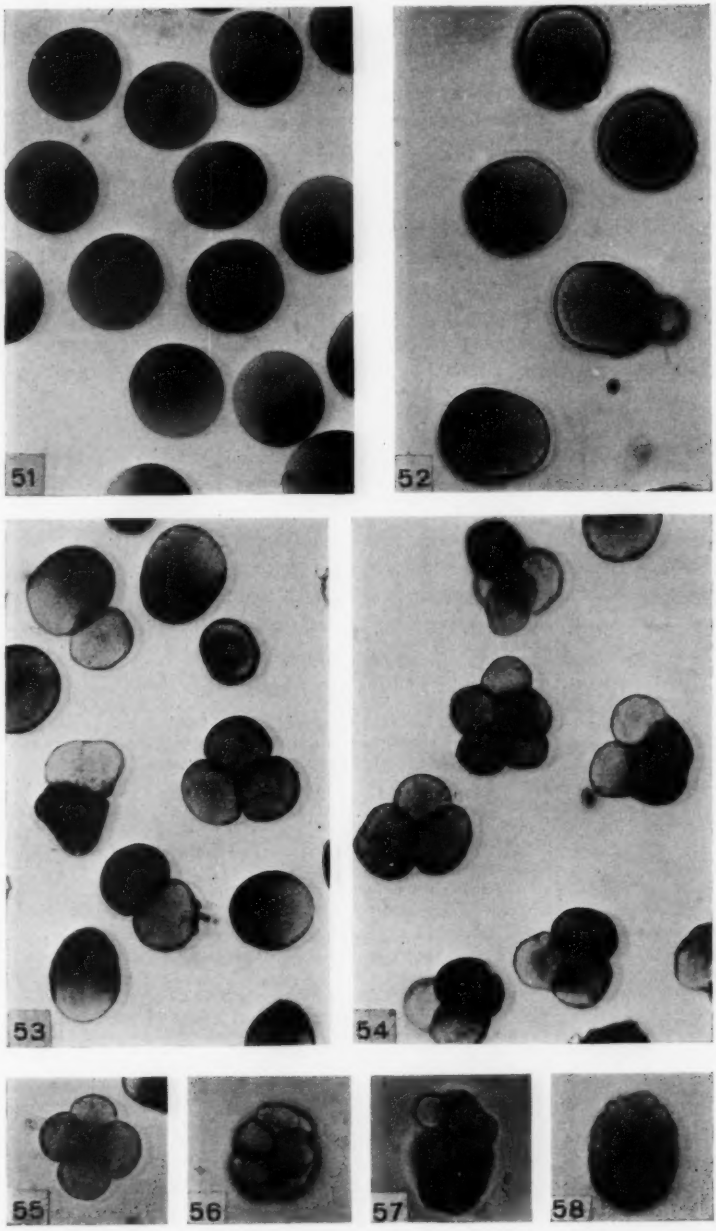

PLATE V

presented (Photographs 55–58). I wish especially to call attention to the very normal early blastula (Photograph 58).

It seems extraordinary that these red halves which have been activated artificially, without ever having had any contact with sperm or substances from the mature female nucleus, should develop better than the red halves, obtained after fertilization, which consist of protoplasm which has been acted upon by sperm in a normal manner, and which in some cases (when centrifuged after the breakdown of the nuclear membrane) has been mixed with the substances of the nucleus. It might be emphasized here that the fact that the germinal vesicle has liberated substances into the cytoplasm before the occurrence of parthenogenetic merogony has not escaped my notice, and this was particularly discussed in my first paper (1936, p. 119). This in itself cannot have any effect on the development of the parthenogenetic merogone since the other red halves (from eggs fertilized, then centrifuged) do not develop and yet have this material also. The interesting feature of parthenogenetic merogony is that an egg will cleave and by successive cleavages form a blastula, without the presence of the mature ♀ nucleus or any substances from it, and without the ♂ nucleus. The protoplasm of a mature egg is necessarily a product of successive generations and it contains materials of the germinal vesicle from within its boundaries

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PLATE VI

Development of Whole Eggs Centrifuged After Fertilization

PHOTOGRAPH 59. Normal 2-cell stage (upper). Egg inside fertilization membrane, in which only white half has cleaved (lower left). Same without fertilization membrane (right). Centrifuged 45 minutes after fertilization. Photographed 2 hours after fertilization.

PHOTOGRAPH 60. Normal micromere stage. Centrifuged just before cleavage. Photographed 2½ hours after fertilization.

PHOTOGRAPH 61. Late cleavage stage, white and red portions distinct. Centrifuged 3 minutes after fertilization. Photographed 4 hours later.

PHOTOGRAPH 62. Blastula soon before hatching. Centrifuged 40 minutes after fertilization. Photographed 6 hours after fertilization.

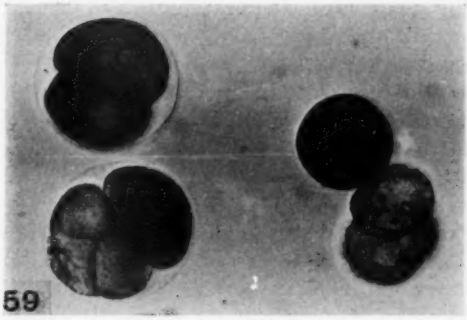
PHOTOGRAPH 63. Whole egg inside fertilization membrane, white portion only developed. Centrifuged just before cleavage. Photographed 6 hours after fertilization.

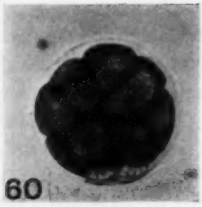
PHOTOGRAPH 64. Both parts develop with nuclei from original diploid nucleus. Centrifuged just before cleavage. Photographed 8 hours after fertilization.

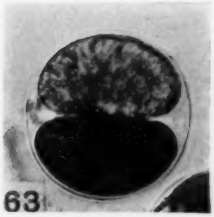
PHOTOGRAPH 65. Similar egg; two nucleate parts about to separate, forming twins. Centrifuged 30 minutes after fertilization. Photographed 9 hours later.

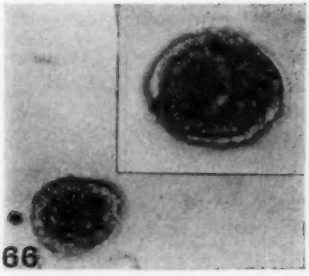
PHOTOGRAPH 66. Similar pair, 3 days old. Larger one is a white blastula, smaller a red gastrula. Centrifuged 40 minutes after fertilization.

PHOTOGRAPHS 67, 68. Two white twins and a red blastula, all with nuclei from original diploid nucleus, forming triplets. Centrifuged soon before cleavage. Photographed 8 hours later.









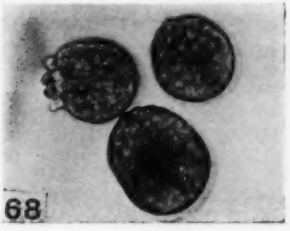


PLATE VI

as well as nutrient materials from without. Certainly the chromosomes and genes which are generally believed to determine its own fate are *not* present in a parthenogenetic merogone.

Since the non-nucleate half-eggs obtained after fertilization behave so differently from those obtained before fertilization and subsequently activated, it is obvious that a change has taken place in the protoplasm on fertilization which changes its developmental potencies as well as its physical and chemical characteristics such as permeability, viscosity and oxygen consumption. This change must take place very rapidly since the non-nucleate halves will not develop even if obtained as quickly as possible after fertilization nor can they be re-activated. The change in rate of stratification of the fertilized and unfertilized eggs also takes place immediately as well as the change in the rate and method of breaking (1933).

In comparison with the amoeboid activity of the red halves of the *Arbacia* egg, obtained after fertilization, it is of interest to refer to the classical work of E. B. Wilson (1904) on *Dentalium*. He found that the non-nucleate portion, obtained by cutting a fertilized egg, and containing the polar lobe, would go through rhythmic phases simultaneously with the cleavage of the nucleate portion and form a polar lobe just as though it were still a part of the complete egg, and even appeared as though it divided into two.

Development of Whole Eggs Centrifuged after Fertilization

Among the eggs broken apart by centrifugal force, there are always, with the forces used, some eggs which have not broken apart, both with and without fertilization membranes. These may develop quite normally like the uncentrifuged egg, even giving off micromeres (Photographs 59, upper egg; 60). Perfectly normal blastulae and plutei are formed. In some cases the stratification remains during cleavage and the egg may still remain elongate if the fertilization membrane has been removed (Photographs 61, 62), resembling the elongate eggs fertilized after centrifugation. In some cases, even within the fertilization membrane, the two parts, the light and the pigmented, may develop independently, and it frequently happens that the white portion cleaves and the red portion does not (Photographs 59, lower left and 63). If without the membrane, the two portions may be only partially separated and start to develop as a whole, both parts being nucleate (Photograph 64). These parts may become free-swimming blastulae and later separate and give a pair of twins, which are different in color, but with the same nuclear make-up (Photograph 65). Several of these pairs were isolated and in all cases after three days, the white twin was a blastula and the

red twin had invaginated (Photograph 66). It would be of interest to know whether these red halves with diploid nuclei would develop better than the fertilized merogones with haploid nuclei which are so difficult to raise. Together with these "twins" occur also "triplets," consisting of a red blastula, and two white blastulae which have apparently developed from the upper portions of the first two blastomeres; these three blastulae are, of course, all nucleate (Photographs 67, 68). My departure from Woods Hole prevented further investigation of these twins and triplets.

Summary

1. Fertilized eggs of *Arbacia* may be broken by centrifugal force into white and red halves similar to the unfertilized egg; the nucleus is in the white half.

2. The white half may develop quite normally through the blastula stage; no normal plutei have been obtained. The first two blastomeres may develop independently forming white twins. Amoeboid forms and loose masses of cells also result from the white half.

3. The red half does not cleave or develop. It may become amoeboid or notched, or form asters, thus indicating activation.

4. It makes little difference at what stage the eggs are centrifuged.

5. The red half cannot be refertilized or activated artificially.

6. Lack of development of the red halves obtained after fertilization is in striking contrast to the development of the red halves obtained before fertilization and subsequently activated artificially (parthenogenetic merogones).

7. Whole eggs may develop normally after centrifuging. They may separate later into 2 or 3 parts, forming nucleate red and white twins or triplets.

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GROWTH AND DIFFERENTIATION OF DAPHNIA MAGNA EGGS IN VITRO

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It is the purpose of this paper to give an account of the development of *Daphnia magna* eggs *in vitro*. To our knowledge the method presented here of growing parthenogenetic eggs of *Cladocera in vitro* instead of in the brood-chamber of the mother represents the first endeavor in this direction. The study was undertaken because in the course of some ultra-centrifuging experiments two years earlier there arose the need of rearing the eggs of this animal in tissue culture slides. The photomicrographs and the brief description of the developmental stages in the course of the growth of the organism outside the brood-chamber of the mother are intended at this time to serve only as a basis for a more critical attack on a large number of possible problems with *Cladocera* in general.

Methods

The animals employed in this work can be traced to Anderson's stock (1932) and this in turn to animals received from Banta's laboratory. This particular species was utilized previously in our laboratory for a period of about five years for other experimental work. The animals were reared only parthenogenetically and they represented genotypically identical individuals derived originally from a single animal.

A number of different media were tried in an endeavor to grow eggs of *Daphnia magna* successfully *in vitro*. A modified Ringer's solution, utilized by Lévy (1927) for physiological work on isolated organs of *Cladocera*, injured the eggs, and in no case was complete development effected. Likewise an endeavor was made to imitate the cultural condition of the brood-chamber by the introduction of varied amounts of the fluid obtained from the brood-chambers of animals in which there were developing eggs. The growth of eggs in such a medium did not show any advantage over a simpler medium consisting of sterile pond water. This is of considerable interest in view of the nutritive rôle which has been ascribed to the fluid present in the brood-chamber (Dearborn, 1908; Birge, 1918). Contrary to expectation,

carefully controlled experiments with eggs reared *in vitro* in a medium consisting of sterile pond water repeatedly showed 100 per cent egg development. Such eggs gave rise to fully developed young in about the same period of time as the eggs developing in the brood-chamber of the mother. These observations establish without any doubt the nutritive sufficiency of materials already stored in each egg at the time of laying, and they also throw some light on the nature and the possible function of the tough membrane which surrounds the developing egg.

A *Daphnia magna* female, the eggs of which were to be removed from the brood-chamber, was allowed to swim for a few minutes in a vessel of pond water which had been rendered sterile by heating (distilled water appears to be injurious to the organism). This procedure freed the animal to some extent from microorganisms which were found to cling to the body and which, when present in the egg medium, interfered with the normal growth of the embryos. The animal was then placed in a depression slide (the type commonly employed for tissue cultures) with a round polished cavity 15 mm. in diameter and 3 mm. deep and the slide was filled with sterile pond water. With the aid of a fine dissecting needle, which was placed over its head, the animal was held in the field of the binocular microscope. Another dissecting needle was applied to the inside of the lower carapace, and the animal was held in place on its side against the surface of the slide. The needle which held the head in place was then transferred to the inside of the upper carapace. The two flaps of the carapace were then spread apart till the upper flap was made to lie against the slide. This procedure exposed the eggs in the brood-chamber. By gently moving the body of the animal back and forth with the dissecting needles, while the flaps of the carapace were still held against the slide, the eggs were made to roll out of the chamber without being subjected to any pressure in the course of the dissection. The remains of the animal were then removed and the water surrounding the eggs was replaced by fresh sterile pond water. The successful removal of eggs from the brood-chamber of the mother without any injury to them determines to a large extent the degree of success in the rearing of the eggs of this animal *in vitro*.

To determine the time required for eggs to complete their development in the brood-chambers of the animals, individual animals were isolated in bottles containing the standard amount of the culture medium (Banta, 1921). On each bottle was recorded the exact time when the eggs were deposited. The animals were reared at 25° C. in a water bath, the temperature of which was controlled to $\pm$ 0.01° C. From repeated observations on animals reared under such conditions, it was

found that with very few exceptions mothers released their young some time during the forty-sixth hour after the deposition of the eggs.

Many series of developing eggs *in vitro* were carried to completion. The length measurements referred to in this paper and the degree of development of the embryos illustrated in the photomicrographs represent observations on a single clutch consisting of 22 eggs. For other observations made simultaneously with this clutch of eggs, there were carried other parallel series *in vitro* and also eggs developing in the brood-chambers of mothers, serving as controls. In an endeavor to perfect the technic of recording the morphological changes in the course of the development, at least 4 other series of photomicrographs were completed and each one of these was accompanied by other parallel series of eggs growing in depression slides and also developing in the brood-chambers of mothers. *Daphnia magna* eggs when reared *in vitro* were found likewise to complete their embryonic development in about 46 hours at 25° C. It was observed, however, that if the growing eggs were subjected to a lower temperature in the course of routine examination or in the course of replenishing from time to time the fluid in the culture slides, there was a delay of several hours in completing the embryonic development. It was found that whenever a young in the tissue culture slide corresponded in development to one just released from the brood-chamber, it extended its caudal spine which up to this time had been closely adhering to the post abdomen (Fig. 15) and immediately began to exhibit more active swimming movements. The extension of the caudal spine and the more active swimming movements which followed were utilized by us as criteria in judging the end point of embryonic development *in vitro*.

Gross Microscopical Examination of Developing Eggs in Vitro

Inasmuch as the embryos are transparent, the rearing of *Daphnia magna* eggs on tissue culture slides enabled us to make direct observations on some of the sequence of events in the course of development of the embryos without the necessity of staining and sectioning.

The parthenogenetic egg of *Daphnia magna* is enclosed in two membranes. The inner membrane is the vitelline and closely adhering to it on the outside, observable even in eggs a few minutes after their deposition, there is a more prominent membrane. Lebedinsky (1891), in a short account of the development of *Daphnia similis,* refers to this membrane as the chorion. These two membranes exhibit a progressive resistance to mechanical injury with lapse of time from the deposition of the eggs. This was repeatedly demonstrated by subjecting the eggs to ultra-centrifuging at various stages in their development. A com-

paratively weak centrifugal force consisting of 1800 times gravity was sufficient to rupture the membranes and bring about a disintegration of newly deposited eggs the moment the ultra-centrifuge was started. Eggs three hours after their deposition withstood the same centrifugal force for several minutes without disintegration. Still older eggs were subjected to a centrifugal force of as much as 50,000 times gravity for several minutes without a bursting of the membrane. In our study three-hour-old eggs were usually chosen for growth in tissue culture slides. Such eggs invariably attained 100 per cent development. Earlier eggs may be reared with equal success, but greater precautionary methods must be employed during the dissection of the mother and in the subsequent handling of the eggs.

The first morphological change noticed in the course of development *in vitro* is the appearance of an invagination in eggs nine hours old, reared at 25° C. This invagination gives the first indication of the prospective cephalic region (Fig. 3). It becomes more prominent in the next three hours of development (Fig. 4). At this stage a vertical constriction appears also at the extreme posterior end (lower edge in the figure), which marks the bilateral symmetrical plan of organization which is to follow.

During the first 18 hours of development the eggs remain nearly perfectly spherical and do not show any increase in size. The clutch of eggs studied under very careful control, measured throughout this period 0.275 mm. in diameter. Figure 5 shows an egg 18 hours old. This egg, although it is of the same size as in the previous stages, shows the beginning of the cephalic appendages and also some slight evidence of the beginning of the abdominal appendages.

The prospective left and right halves of the future organism are well marked in embryos 21 hours old (Fig. 6). At this stage the embryos measured 0.337 mm. in length in contrast to 0.275 mm. which characterized the length of all the previous stages up to this point. This represents an initial increase in length of about 60 μ. At this same stage there appears also mid-dorsally at the cephalic region a blastodermic thickening, which constitutes the first external evidence of brain development. The prospective cells which are to form the brain are definitely recognizable earlier in prepared slides. In eggs 24 hours old (Fig. 7) this organ is unmistakable when viewed grossly under the microscope.

In embryos 27 hours old (Fig. 8) there comes into prominence, dorsal to the prospective brain, a mass of granular substance at first grayish in appearance, then, with subsequent development becoming brown and finally black. This granular material, which at first is represented by a single mass, in the 30-hour embryo, is seen to differentiate into two

well-established masses now brownish in color, representing the material for the development of the eye (not in focus in the photomicrograph, Fig. 9). This double mass is clearly shown in Fig. 10, representing an embryo 33 hours old. Anterior to the eye masses there is visible also the dense material for the prospective ocellus. The eye in a fully developed individual is represented by a single organ placed in the front of the head, composed of ommatidia. It is interesting to note that this cyclopian eye of *Daphnia magna* has embryologically a double origin. Whether or not the sequence of events involved in the development of this organ has phylogenetic significance remains to be investigated. So gradual and orderly is the fusing of the eye masses into a single structure that we are utilizing this process in our laboratory as a criterion in judging the stage of the development of the embryo *in vitro*, also in predicting the time of the release of the young from the brood-chamber.

The first sign of movement of the body was observed in embryos 30 hours old. This was characterized by slow lateral expansions and contractions of the entire body, resembling massaging motions. There were also observed in the embryo at this stage a few irregular heart

PLATE I

Photomicrographs of *Daphnia magna* eggs and embryos in the course of their development *in vitro*. Figures 1, 2, 3, 4 represent prints from negatives. Figures 5–12 represent prints made by direct projection of the living material on Defender Velour Black paper utilizing a Bausch and Lomb vertical microscopical slide projector. They are negatives. The magnification is the same throughout. × 117.

FIG. 1. Egg 3 hours after deposition.

FIG. 2. Egg 6 hours old showing the beginning of the formation of a clear zone around the periphery.

FIG. 3. Egg 9 hours old showing the first invagination. The interior light areas in Figs. 1–3 are oil droplets.

FIG. 4. Egg 12 hours old showing the chorion and the first demarcation of the future cephalic portion. A constriction at the extreme posterior end marks the beginning of the bilateral symmetrical plan of development.

FIG. 5. Egg 18 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIG. 6. Embryo 21 hours old showing further demarcation of the cephalic and abdominal appendages and also a more pronounced revelation of the bilateral symmetrical development.

FIGS. 7, 8, 9. Embryos 24, 27 and 30 hours old respectively, showing further developmental differentiations.

FIG. 10. Embryo 33 hours old, showing the early appearance of the two eye pigment bodies and posterior to them the ocellus, the black bodies surrounded by a clear area, centrally placed in the cephalic region. These bodies are not in focus in the earlier stages.

FIG. 11. Embryo 36 hours old, showing the further differentiation of the two eye masses and the ocellus, the white bodies at the extreme anterior end.

FIG. 12. Side view of an embryo 39 hours old after infolding of the cephalothorax.

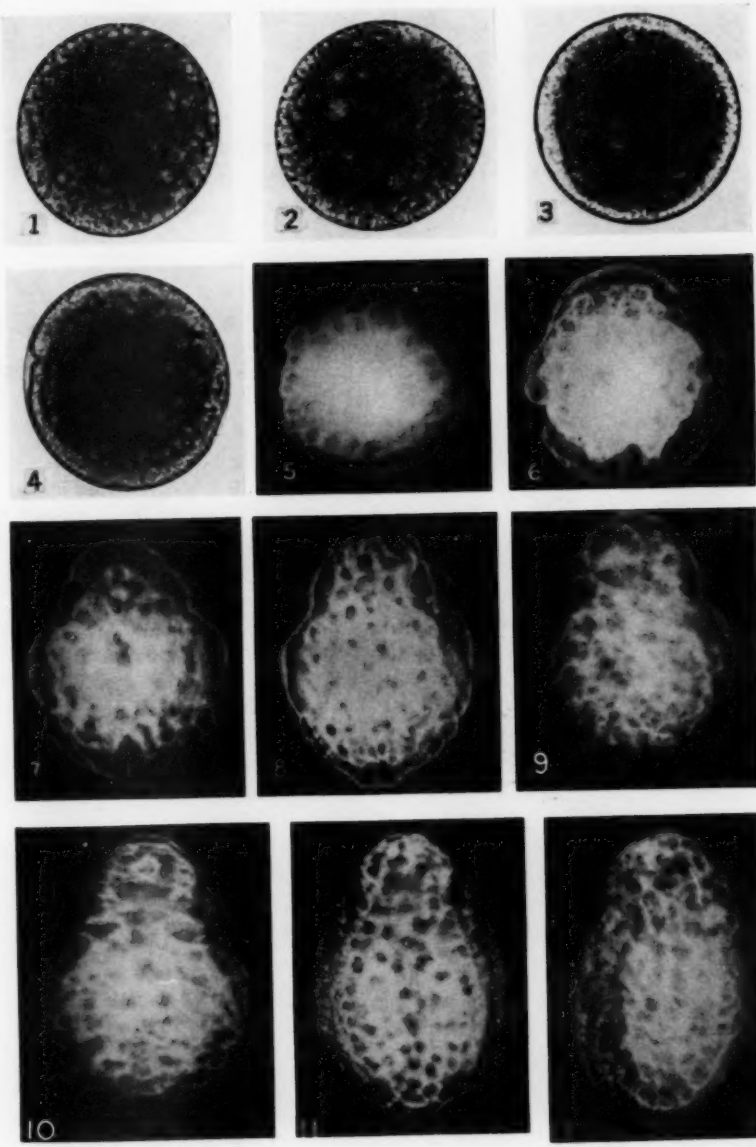

PLATE I

beats per minute. Five hours later, the heart beats increased to about 20 per minute and in 36-hour embryos the rate became 32 per minute. With the further differentiation of the organism, it became more regular and more rapid. Fully formed young when in their second instar show a heart-beat rate of about 180–200 per minute but this varies greatly with the temperature. The observations recorded here were all made at 19° C. ± 1° C. It is evident, therefore, that the embryonic rate of the heart beat of this organism is less than that in the adult.

The increase in body length in the course of the embryonic development of *Daphnia magna in vitro* is not continuous. There are periods during which no increase in length can be observed, during which time undoubtedly other changes are taking place. As already pointed out, the first increase in body length of about 60 μ was seen in embryos 21 hours old. In the next 3 hours of development there was an additional increase of about 40 μ, and in embryos 27 hours old the body length became 0.41 mm., representing a further growth of about 30 μ over the previous stage. After this there followed a 9-hour period during which the embryos increased in width rather than in length (30, 33 and 36-hour stages). This is more clearly demonstrated in Fig. 10 showing an embryo 33 hours old, just before the infolding of the cephalothorax has begun. After the 36-hour stage and for the next 9 hours there was again observed a gradual increase in body length varying from 20 μ to 60 μ for each 3-hour period of development. The most pronounced increase in body length took place during the hour just previous to the completion of the embryonic development. It appears that with the extension of the caudal spine (Fig. 14) there is also freed a portion of the posterior carapace heretofore held in place. This may account for the apparent sudden increase in the length of the embryo of about 180 μ in one hour (excluding the caudal spine) which is considerable considering the previous increments of growth. This sudden increase in growth may be also due to imbibition of water at this period, a phenomenon so characteristic in fully grown individuals between their successive instars. In some *Daphnia* Miss Rae Whitney of Brown Uni-

PLATE II

Photomicrographs of *Daphnia magna* embryos in the course of their development *in vitro*, made by direct projection of the embryos on Defender Velour Black paper. They are negatives. The magnification is the same throughout. × 130.

FIG. 13. Embryo 42 hours old showing the eyes laterally placed, and the caudal spine closely adhering to the post-abdomen.

FIG. 14. Embryo 45 hours old—one hour before it emerges as a fully developed young. The caudal spine is only partially extended.

FIG. 15. Embryo 46 hours old representing a fully developed young. The caudal spine is now fully extended.

PLATE II

versity finds a pre-natal molt at this stage of the development (unpublished observations).

After the extension of the caudal spine (Fig. 15) the individuals resemble in every respect young just freed from the brood-chambers of mothers. They immediately become more active, utilizing to a fuller extent in their locomotion the antennae and other appendages, thus completing a history of embryonic development from eggs to independent free-swimming organisms in about 46 hours. The individuals at this stage measure about 0.74 mm. in body length. When such individuals are transferred to bottles containing a standard amount of culture medium (Banta, 1921), but at first in a more diluted form, they, too, become mature and in time produce other young.

Summary

1. The parthenogenetic eggs of *Daphnia magna* may be reared in sterile pond water on tissue culture slides, completing their embryonic history from egg to free-swimming independent organisms in about 46 hours.

2. The eggs of this animal are self-sufficient with regard to the nutritive materials already stored in them at the time of their deposition. Such eggs are transformed into fully developed young *in vitro* in about the same period of time as that required for the development of eggs in the brood-chamber of the mother.

3. Fully developed young reared *in vitro*, when transferred to bottles containing the standard amount of culture medium, become in time sexually mature and produce young.

4. A brief description is given of the sequence of events in the course of the development of the embryos reared on tissue culture slides, as observed by gross microscopical examination.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XII. Some Physiological Changes in Eggs the Embryos of Which Have Been Destroyed by X-Irradiation [1]

JOSEPH HALL BODINE, LOREN D. CARLSON AND O. MALCOLM RAY [2]

(From the Zoölogical Laboratory, State University of Iowa)

Investigations have been conducted on the effect of x-irradiation on the morphology, respiration and enzyme (protyrosinase) formation in the egg of the grasshopper, *Melanoplus differentialis* (Evans, 1934; 1935; 1936; 1937; Boell, Ray and Bodine, 1937; Ray, 1938). By the fifth day of development at 25° C. differentiation has progressed to such a degree that the embryo may be dissected from the egg (Slifer and King, 1934). An x-irradiation of 1000 r given the egg on the sixth day after laying will cause the destruction of the embryo proper leaving the yolk and serosa cells intact (Evans, 1936; Ray, 1938). This permits a study to be made on some of the physiological activities of these eggs from which the embryo has been thus removed.

The following three criteria of physiological activity in these eggs were used: (1) Respiration: The respiration of the whole egg as well as of the contained embryo has been reported (Bodine and Boell, 1936). For the first 15 to 20 days after laying, the rate of O_2 uptake increases; then it declines to a minimum by the twentieth to twenty-fifth day and remains constant during the blocked or diapause state. This period of inactivity (diapause) may be terminated by exposure to 5° C. and when the eggs are again placed at 25° C. there is a rapid increase in the rate of respiration until the grasshopper hatches on the eighteenth to twentieth day. The respiration of the embryo is, at first, lower than that of the intact egg, but later it accounts for a considerable part of late prediapause and postdiapause respiration (Bodine and Boell, 1936). (2) Formation of the enzyme protyrosinase: This enzyme in the normal egg begins to form on the tenth day and by the twentieth day has reached a maximum which is maintained during further development (Bodine, Allen and Boell, 1937). The remarkable resistance of this enzyme to x-irradiation during its formation has been reported (Ray,

[1] Aided by a grant from the Rockefeller Foundation for work on physiology of the normal cell.

[2] Now at the State Agricultural College of North Dakota.

1938). (3) The ontogenetical change in potency of the naturally occurring activator of protyrosinase: This has recently been investigated by Bodine and others (1939).

Material and Methods

Experiments have been carried out during three successive years, 1937 to 1940, and different lots of eggs used each year. In each lot the eggs were collected on the day of laying, carefully selected and divided into two groups. One of these was irradiated on the sixth day of development; the other kept as a control. The roentgen apparatus used was a double cross-arm mechanically rectified unit energizing a Coolidge broad focus universal air-cooled tube at 130 kv.; and 5 ma. The eggs were spread on several layers of damp towelling and irradiated at a distance of 53 cm. for 20 minutes. Previous calibration with an ionization chamber showed a delivery of 52 r per minute at this distance giving the eggs a total dosage of 1040 r.[3]

The eggs were kept on moist sand at 25° C. during development and placed at 5° C. for 3 months to terminate the diapause. Control eggs were examined at various stages and sorted according to morphological development as reported by Slifer (1932). Care was taken to give the irradiated eggs the same treatment as control eggs. Daily examinations were also made of the experimental material to determine possible injury.

Oxygen consumption of control and irradiated eggs was determined by placing the eggs in lots of 25 or 50 on moist filter paper in standard micro-Warburg manometers.

Tyrosinase activity was measured in egg brei. This was prepared by grinding 100 eggs in a glass mortar and diluting to a volume of 5 cc. with a NaCl solution buffered to pH 6.8 (50 cc. 0.45 per cent NaCl — 25 cc. M/15 Na_2HPO_4 — 25 cc. M/15 KH_2PO_4). The amount of enzyme in this brei was determined in standard Warburg manometers by activating the enzyme with 0.1 cc. 5 per cent sodium oleate and using 0.3 cc. 0.4 per cent tyramine hydrochloride as a substrate. The activity of the enzyme was determined from the rate of oxygen uptake and expressed as $1/T \times 10^3$ where T is the time of half oxidation of the substrate (194 μ l. O_2).

The amounts of natural activator occurring during ontogenesis were determined by the "brei method" described by Bodine et al. (1939). In those cases where the protyrosinase content was low more was added in the form of a standard protyrosinase solution termed B_1 (Bodine et

[3] The authors are indebted to Dr. T. C. Evans for x-raying eggs and for making cell counts.

al., 1939). The amount of activation is represented in terms of rate as shown for tyrosinase activity.

Results

Morphological changes in the eggs were followed daily by removing 10 to 20 eggs at random and inspecting them after dissection under a widefield binocular microscope. In no case was a formed embryo found

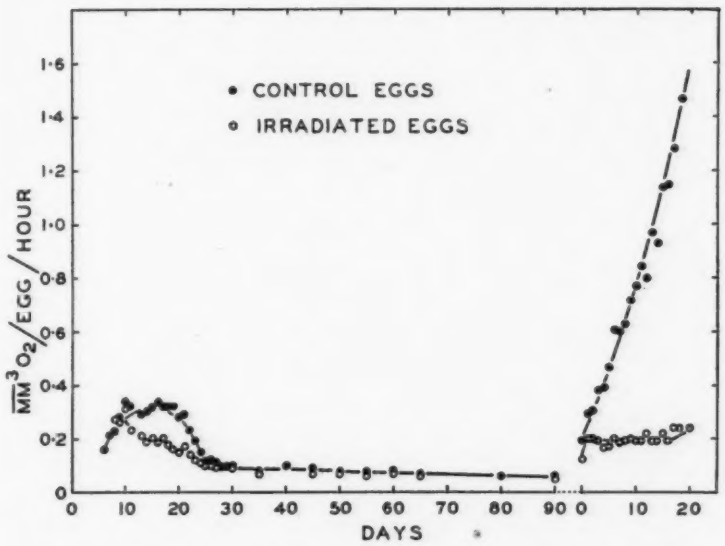

FIG. 1. Shows the rate of oxygen consumption per egg in cubic millimeters per hour. Ordinate, rate of oxygen uptake; abscissa, time in days at 25° C. since laying followed by the time in days at 25° C. after termination of the diapause by exposure to 5° C. for 3 months. ●, control eggs; ○, irradiated eggs. Each point is an average obtained from 3 manometers over a 2- to 3-hour period. Twelve thousand eggs were used in these determinations. Other lots used gave similar results.

in the irradiated eggs. By use of smears of the egg contents counts of yolk and serosa cells of both control and irradiated eggs were made. No appreciable differences in appearance or cell counts of experimental and control eggs were detected. Traces of embryonic cells in irradiated eggs occur only for a short period following irradiation. From both gross and microscopic examination one may conclude that the visible effects of irradiation are confined to the embryo proper and not to the

yolk or serosa cells. No evidence of the presence of an embryo in irradiated eggs can be found. Further data concerning morphological changes due to x-irradiation are to be discussed in a subsequent communication from this laboratory by Dr. Titus Evans.

The O_2 consumption curves for both irradiated and control eggs are shown in Fig. 1. The respiration of the irradiated eggs increased slightly during the first 3 days after irradiation and then slowly decreased

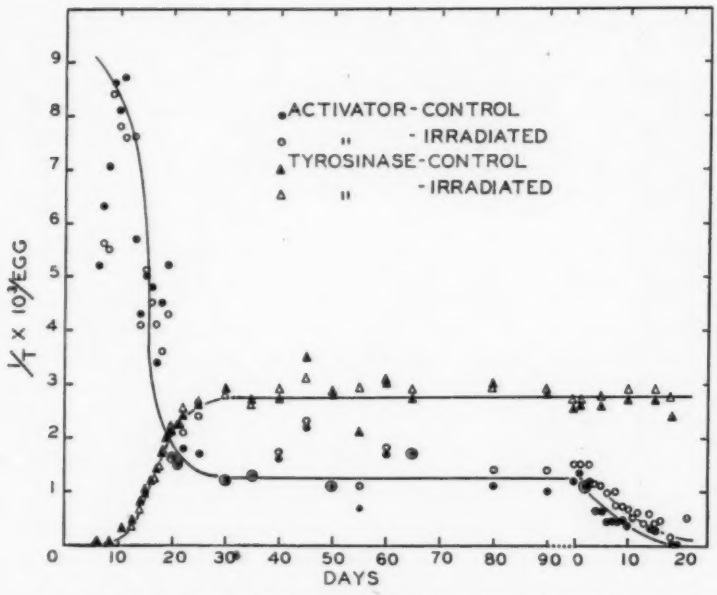

FIG. 2. The formation of protyrosinase and the amount of naturally occurring activator during ontogenesis. The ordinate shows the specific reaction rate per egg. The rate is the reciprocal of the half oxidation period of the substrate multiplied by 10^4. The abscissa is the time in days at 25° C. as described under Fig. 1. ▲, protyrosinase in control eggs; △, protyrosinase in irradiated eggs; ●, activator in control eggs; ○, activator in irradiated eggs. Twelve thousand eggs were used in these determinations.

up to the time when the control eggs entered diapause. The O_2 consumption rates of both irradiated and control eggs are similar during diapause. When subjected to 5° C. for 3 months, diapause is terminated in both experimental and control eggs since when they are returned to 25° C. increases in their rates of O_2 consumption are noted. By reference to Fig. 1, it can be seen that respiration in control eggs increases markedly during postdiapause up to the time of hatching. Irradiated

eggs, on the other hand, increase in O_2 consumption rates for a period of 3 days and reach a maximum at which they remain throughout the course of the experiment.

The growth of the enzyme, protyrosinase, in both control and irradiated eggs is given by curves in Fig. 2. An examination of these shows no difference in the enzyme production in the two types of eggs and these results are a direct confirmation of those described by Ray (1938). Changes in potency of the naturally occurring activator of protyrosinase are also given in Fig. 2. It will also be noted that no significant differences in the type of curve for irradiated and control eggs are apparent. The general shapes of the curve for natural activator are similar to those previously reported (Bodine, Ray, Allen and Carlson, 1939). After the termination of diapause, it will be observed that the decline in potency of the activator in the irradiated eggs is less than that of the control eggs. No explanation of this difference is at hand.

Discussion

It may be well to review briefly the early stages in the development of grasshopper eggs at 25° C. to better appreciate the significance of the results of the present experiments. At the end of the first day after laying, cleavage has progressed and the resulting nuclei are found scattered in the yolk with a few in the peripheral protoplasm. On the second day there are many nuclei, some in the yolk, some in the peripheral layer. During the third and fourth day, the germ band forms and by the fifth day, the embryo is large enough to dissect from the egg (Slifer and King, 1934). On the sixth day, the cells at the periphery (serosa) are secreting the cuticle. The extra-embryonic cells cease mitotic activity after the fourth or fifth day of development and after the seventh day there is no significant change in the character of the yolk and serosa cells (Evans, 1940). The marked resistance to x-irradiation of the serosa and yolk cells permits the destruction of the embryo tissue without damaging either yolk or serosa (Evans, 1936; Ray, 1938). The physiological processes which are evidenced in the measurements made on irradiated eggs are, therefore, most likely accomplished by the yolk and serosa cells. Preliminary experiments show that the respiratory quotient is not markedly changed in the irradiated eggs though the oxygen uptake during the period of active development is somewhat less than that of the control. Bodine and Boell (1936) have shown the relationship between respiration of embryo and whole egg during development and it seems interesting to note that in the present experiments, the periods where the O_2 uptake of the control eggs exceeds that of the

irradiated eggs corresponds to the times when the embryo respiration is the major part of the total respiration. They also noted that the initial increase in oxygen uptake when diapause or the block to normal development and cell activity is removed is for the most part due to yolk cells and extra-embryonic material. A rise in oxygen consumption of irradiated eggs (without embryos) occurs at this time, and in fact, it is the period in which the total postdiapause increase in respiration occurs.

These investigations, then, on the rate of oxygen consumption, an enzyme formation (presumably a protein) and the decrease in potency of the activator of this enzyme (a lipoidal constituent) seem to show that definite metabolic processes occur in the yolk and serosa cells even in the absence of an embryo and that these cells may play an important part in the general metabolism of the whole egg. Evidence from the rate of respiration (Fig. 1) and from the decrease in potency of the activator (Fig. 2) seems to demonstrate that these cells in the egg in the absence of the formed embryo appear to become blocked during development and exhibit the general characteristics found in the normal development of the grasshopper egg, i.e. a prediapause, diapause and postdiapause periods.

Further investigations in the morphology and physiology of these eggs to bring out fundamental relationships seem desirable.

Summary and Conclusions

1. The embryos in the egg of the grasshopper (*Melanoplus differentialis*) can be totally destroyed by an x-irradiation of 1000 r on the sixth day of development.

2. Eggs deprived of an embryo in such a manner have been studied in regard to their oxygen consumption, the formation of protyrosinase, and the amount of natural activator of this enzyme.

3. The respiration of the irradiated eggs differs from that of the controls during the active stages of development. The oxygen uptake of the irradiated eggs gradually falls during prediapause until it reaches the diapause level. An increase in the rate of respiration of these eggs occurs during the first 3 days of postdiapause.

4. The enzyme protyrosinase is formed in the same manner in both experimental and control eggs.

5. The potency of natural activator in irradiated eggs does not differ from that of controls until postdiapause when the decrease in potency is slightly less in experimental eggs.

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THE DISTRIBUTION OF ELECTROLYTES IN PHASCOLOSOMA MUSCLE

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In recent years considerable attention has been paid to the distribution of electrolytes in tissues, particularly muscle and nerve. It has been recognized, of course, that the total electrolyte content of such a tissue as muscle must be distributed between at least two phases, one being roughly the cellular phase, the other extracellular.

That protoplasm is a solution of electrolytes, among other things, has been amply demonstrated by observations on conductivity. Gross analysis of masses of cells and tissues shows that these electrolytes must be largely of a type with inorganic cations and organic anions (see Fenn, 1936). Many of the organic substances in cells have been described, but until recently little attention has been paid to the distribution of the inorganic constituents.

The general principle that, in many tissues composed of highly differentiated cells, chloride does not penetrate into the cells (Fenn, *loc. cit.* See also Amberson et al, 1938) is very useful, since it allows calculations to be made of the approximate amounts of other inorganic constituents present in the extracellular space and hence, cellular concentrations, if the total concentration in the tissue is known. The method of study is very simple, involving soaking the tissues in chosen solutions and then analyzing them for chloride and the other elements in question. The data then are treated on the assumptions that all electrolytes of the external medium are in simple equilibrium with the extracellular space (chloride space) and that the total amounts of metallic elements found in the ash may be regarded as a source of positively charged ions, either within or without the cells.

A recent study (Steinbach, 1940) dealing with chloride, sodium, potassium and calcium in *Thyone* muscle has been reported. The results given in the present paper are concerned with similar experiments on another invertebrate smooth muscle, the retractor muscle of the marine annelid *Phascolosoma.*

The structure of these muscles has been described by Olson (1940).

Methods

The methods used were identical with those reported previously (Steinbach, 1940). The muscles were excised with as little injury as possible and immersed in fifty to one hundred times their weight of solution. Solutions used were either normal sea water, isotonic salt solutions, isotonic buffered sucrose solutions or mixtures of these as indicated. All solutions had a pH of 8.0 to 8.5. The experiments were carried out at room temperature.

Results

Phascolosoma muscles swell when transferred to sea water and their composition changes accordingly. Table I gives average figures for

Table I

Analysis of *Phascolosoma* muscle, fresh (column 1) and treated with sea water for several hours (column 2). Column 3 gives the analysis of *Phascolosoma* body fluid and column 4 of Woods Hole sea water.* Figures in brackets give number of analyses averaged. Concentrations in milliequivalents per hundred grams.

Element	(1) Fresh	(2) Soaked	(3) Body fluid	(4) Sea water
Na	12.2 [3]	14.9 [6]	37.8 [2]	44
K	10.6 [4]	9.0 [2]	3.8 [2]	0.9
Ca	0.85 [4]	1.14 [4]	2.1 [3]	1.8
Cl	9.1 [8]	16.0 [9]	43.0 [2]	51
Relative weight	100	119		
Dry weight %	22		9	
Ash weight %	2.1			

* Sea water is not constant in composition from year to year. The figure for chloride, for example, is from data of 1938. In 1939 the chloride was 2 to 3 per cent lower.

Na, Cl, K and Ca contents of muscles freshly removed from the body and muscles that have been soaked in sea water for several hours. Figures are also included in the table showing the concentrations of these elements in body fluid of *Phascolosoma* and in Woods Hole sea water.

The change in chloride of the muscles can be almost entirely accounted for by assuming that the change of weight represents an increase in the extracellular space. Sodium is present in fresh muscle slightly in excess of chloride. In soaked muscles the situation is changed and most of the chloride can be accounted for as sodium

chloride. A little potassium is lost on soaking and calcium increases slightly.

The main purpose of this work was to follow the loss or gain of cellular electrolytes, using the chloride space (ratio of Cl inside to Cl outside) as a measure of the extracellular space of the whole tissue. In order to do this, it was first essential to show that chloride in the tissue bore a simple linear relationship to the chloride concentration of the medium. Muscles were soaked in various dilutions of sea water with isotonic sucrose solutions and then analyzed. A few experiments

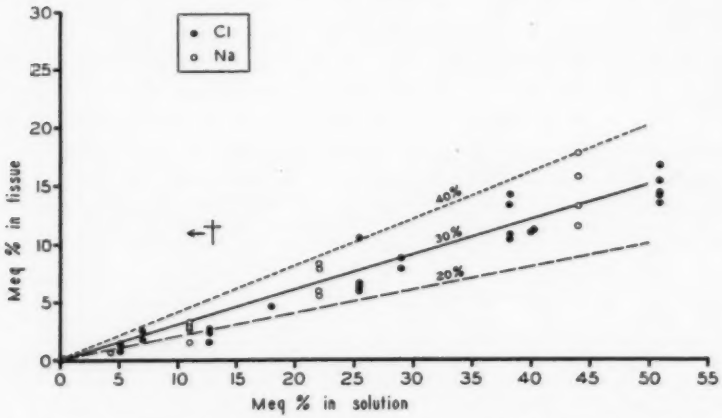

FIG. 1. Chloride (●) and sodium (○) concentrations in *Phascolosoma* muscles plotted against the same elements in solution. The concentrations in the solutions were altered by diluting normal sea water with isotonic buffered sucrose solutions. Tissues soaked 2 to 4 hours before analysis. The cross indicates the minimal salt concentration for maintaining irritability (response to electrical stimulation). All concentrations in milliequivalents per hundred grams solution or final wet weight of tissue. Straight lines represent calculated values for chloride space as indicated. Each point is a single determination on several muscles.

were also done in which normal sea water was diluted with an artificial sea water (Allen's formula) made up with nitrates instead of chlorides. The results (Fig. 1) show clearly that chloride in the muscle does bear a simple linear relationship to chloride of the medium and hence the assumption of an extracellular chloride space is probably correct. On this basis, about 30 per cent of the whole tissue is extracellular space. Determinations of sodium were made on muscles similarly treated and since sodium appears to be extracellular in soaked muscles the results are included in Fig. 1.

The behavior of potassium in the muscles was studied by immersing muscles in solutions of varying potassium content made by diluting either an artificial potassium-free sea water (Allen's formula) or normal sea water with isotonic KCl solution. For each solution being tested, two batches of muscles were used, the one to be analyzed for

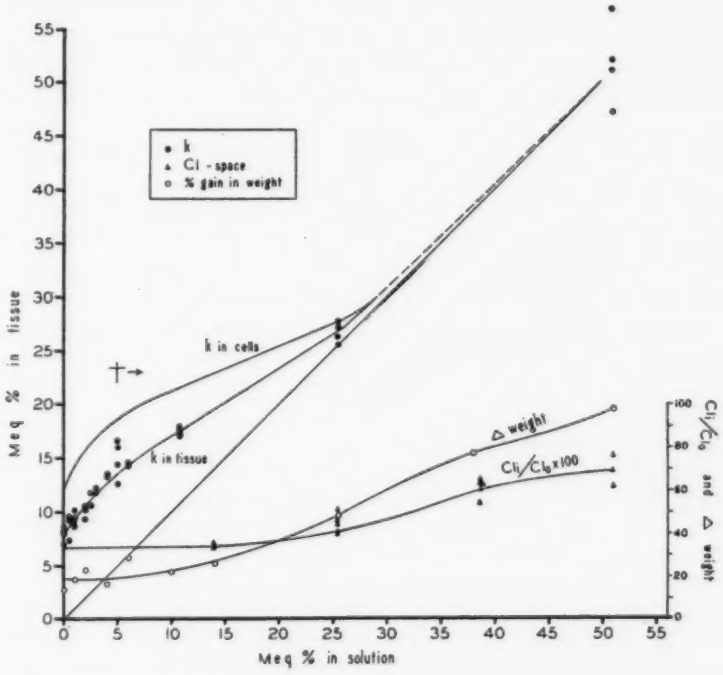

FIG. 2. Potassium concentrations in *Phascolosoma* muscles (●), weight changes (○) and chloride space (▲) plotted against potassium concentrations in the external medium. Tissues soaked 6 to 8 hours. Solutions made up by diluting sea water with potassium chloride solutions. Cross indicates highest potassium concentration at which irritability is maintained. The curve for potassium in the cells represents potassium concentrations calculated as outlined in Table II. Concentrations in milliequivalents per hundred grams solution or final wet weight of tissue.

potassium, the other for chloride. Initial and final weights were recorded. The results are reported in terms of milliequivalents per hundred grams (meq per cent) final wet weight of tissue. Figure 2 summarizes the results. Potassium is lost from the tissue to K-free

solutions and is gained in increasing amounts as the potassium content of the medium is increased. Potassium is first concentrated in the tissue but the amount gained for each increment in potassium of the medium becomes less with higher concentrations until, when the solution is about 0.25 M in potassium, tissue and solution have nearly equal concentrations. Further increases in potassium of the medium do not appear to disturb this last relationship. The potassium concentration in the cells was estimated by subtracting the potassium present in the chloride space ($Cl_i/Cl_o \times$ K-conc. of solution) from the total potassium and dividing the results by the cell space ($1 - Cl_i/Cl_o$). Values

TABLE II

Calculated figures for potassium concentrations in the cells (column 5) and cell volume (column 6). Calculations performed using figures read from the smoothed-out curves drawn through the experimental points of Fig. 2. Concentrations in milliequivalents per hundred grams.

Relative weight (column 2) $= \frac{\text{final weight}}{\text{initial weight}}$. $Cl_i/Cl_o = \frac{\text{chloride in tissue}}{\text{chloride in solution}}$.

K concentration in cells (column 5) calculated by subtracting product $Cl_i/Cl_o \times$ K in solution from K concentration of tissue and dividing by cell space ($1 - Cl_i/Cl_o$). Cell volume calculated by multiplying relative weight by cell space. Average chloride of all solutions assumed to be 52 meq per cent.

(1) Conc. K solution (fresh muscle)	(2) Relative weight tissue	(3) Conc. K tissue	(4) Cl_i/Cl_o	(5) Conc. K cells	(6) Cell volume
	100	10	0.17	11.8	83
0	119	8	0.33	11.9	80
5	120	14	0.33	18.5	80
10	123	17	0.33	22.0	82
15	128	20	0.35	22.8	83
20	133	23	0.37	24.7	84
25	139	26	0.42	26.7	81
35	171	35	0.57	34.8	73
50	199	50	0.67	50.0	66

for these calculations were read from the smoothed-out curves of Fig. 2 and the results are plotted in Fig. 2 (upper curve). These results merely indicate that the concentration of cellular potassium is considerably higher than the potassium concentration of the medium at first, the difference decreasing with increasing concentrations until when the solution is about 0.25 M in potassium the two phases are equally concentrated. Since the cells contain most of the dry substance, this means that, on the basis of water content, the cells always contain a higher concentration of potassium than is found in the external medium.

The calculations of cellular potassium concentrations are calculations based on the assumption that chloride does not penetrate the living cells. That is, cellular potassium concentration figures really represent the amount of potassium present in excess of chloride and presumably occupying space not penetrated by chloride. The mechanism for partitioning the elements in this fashion may or may not be the cell membrane but whatever it is, it has the interesting result that "cell space" remains almost constant in spite of the fact that the muscle swells to almost double its original size. This fact is brought out clearly by the figures given in Table II. These figures are calculated from data read from the smoothed-out curves of Fig. 2. Cell size (volume of muscle not containing chloride) is calculated by multiplying the final relative weight (initial weight = 100) by the cell space ($1 - Cl_i/Cl_o$). From the figures shown it is apparent that these muscles can increase in weight by as much as 40 per cent and more than double the chloride space without any appreciable change in the calculated cell volume. It would seem rather doubtful that this actually means a constant cell size. A more liberal and probably more correct interpretation of the results would be that the protoplasm, due to its whole chemical and physical structure, represents non-solvent space as far as chloride is concerned. Permeability might be a factor in this but modified Donnan equilibria or other chemical equilibria must also be important. This interpretation probably holds also for *Thyone* muscles but is not needed so obviously since those tissues do not swell to any great extent in solutions of high potassium concentration.

The calcium balance in the tissue was studied by methods similar to those used for potassium, except that dilutions were made with calcium chloride solutions. The results of the studies are shown in Fig. 3. There is a nearly linear relationship between the calcium of the muscle and calcium of the solutions except that a small residue remains in the muscle even after prolonged soaking in calcium-free solutions. The slope of the line drawn through the experimental points is greater than that of Fig. 1, showing that some calcium is probably entering the cells. On the other hand, the results also show that calcium is never as concentrated in the cells as in the external medium so long as the outside concentration is above that of normal sea water.

As the calcium of the medium is increased, swelling of the tissues becomes less and the chloride space increases. This may indicate that excess calcium actually causes a shrinkage of the cells but it might also show that some cells are being destroyed. Calculations show that not only is total cellular calcium lower in tissues in high-calcium solutions,

but the concentration of calcium in the cells is also lower. This is similar to the situation found in *Thyone* (Steinbach, 1940) and shows that there must be some change in the cells under the influence of high external calcium so that less calcium can enter or stick to them.

Calculations similar to those made for potassium show that as the calcium of the medium increases the cell size decreases. This is ap-

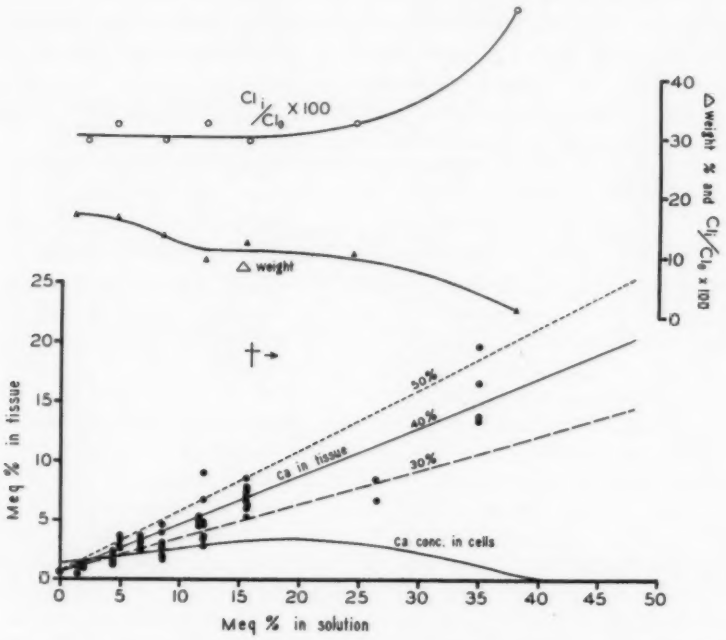

FIG. 3. Changes in *Phascolosoma* muscles as the calcium concentration of the medium is altered. Symbols, concentrations and methods of calculation as in Fig. 2.

parent from inspection of Fig. 3. The total weight of the tissue is less when the calcium is high outside and the chloride space increases; therefore there must be a decrease in cell (non-chloride) space. This may mean either shrinkage or destruction of cells. These results do not differentiate between the two possible changes.

Discussion

The concept of the chloride space of muscle tissue is a useful one. The evidence is good that under normal conditions all of the chloride is extracellular in frog striated muscle, and is quite satisfactory for other vertebrate tissues as well (see Manery and Hastings, 1938). Normal *Thyone* muscle appears to contain chloride only in the extracellular spaces and the present evidence would show that the same holds true in *Phascolosoma*. Both these latter muscles would be classed as smooth muscles; thus another similarity is shown between smooth and striated muscle tissue.

The constancy of the chloride space under normal conditions and the regular way in which it changes with various experimental procedures can be regarded as evidence that there is some definite exclusion of chloride from part of the muscle volume. It is, however, a little difficult to conclude that the cells of *Phascolosoma* muscle change as little in size as is shown in Table II, when the whole muscle doubles in weight. It seems more probable that the partition of chloride is not a definite exclusion by a discrete membrane but depends upon a number of chemical and physical factors so balanced that there is a certain volume of tissue that is, for want of a better term, non-solvent space with respect to chloride. This non-solvent space for chloride probably is nearly equivalent to cell space in normal tissue. But in greatly swollen tissue this may or may not be true. Some start has been made toward a histological study of muscles swollen in KCl and the results, while inconclusive, would indicate that the cells do not stay the same size under all these conditions. It probably is best, then, to relate chloride in swollen muscles to the organic content of the protoplasm rather than to size of cells. On this basis the calculated cell sizes given in Table II would then show that the organic (dry weight) constituents of the cells were still present, occupying space and influencing chemical equilibria as they do in normal cells.

On this basis, the concentration of potassium in such a manner that there is always more potassium inside cells than outside, figured per unit weight of water, probably represents potassium entering into some special equilibrium with the protoplasmic constituents rather than being locked within a membrane. The two mechanisms, of course, have the same effect, but a membrane explaining the results reported here would have to possess most remarkable properties.

Several problems arise in connection with the potassium studies. Potassium enters the muscle cells so that there is always more potassium

inside than outside. There can be little sodium within the cells and other inorganic cations are present in low concentration. Since chloride does not enter, there is then the problem of accounting for the mode of existence of the potassium. The cells are obviously in osmotic equilibrium with their normal environment and yet the internal potassium content can be doubled without a change in calculated cell size. Potassium then enters where there was no potassium before, no swelling occurs and there is no common cation exchanged or anion entering. The only possible solution would seem to be the creation of new electrolytes, probably organic acids, from non-electrolytes previously active only in maintaining osmotic balance. There is at present no clue as to the nature of this process or how it might take place.

The calcium balance of the cells presents as much of a problem as the potassium balance but here the mechanisms concerned allow a partial penetration of calcium, not a concentration of the element. Except with very low external calcium, there is always less of the element in the tissue than in the medium. Furthermore, the changes in calcium content of the cells indicate that the ability to take up calcium from the environment is lowered by treatment with excess calcium in the medium. In view of the demonstrated importance of calcium in surface phenomena (see Heilbrunn, 1937), it seems probable that calcium never penetrates completely within the cell but is stopped by some surface equilibrium condition.

Summary

An analysis is presented of the major inorganic constituents of *Phascolosoma* muscle and body fluid. As is usually found in muscle tissue, potassium is more concentrated in the tissue than in the body fluid or in sea water. Calcium is in about the same concentration while sodium and chloride are in the tissue in lower concentrations than they exist in the external medium.

Sodium and chloride appear to be extracellular in these muscles, most of the potassium and some calcium are intracellular. Parallel analyses of muscles for different elements show that the cells can take up considerably more potassium than they normally contain without an increase in chloride. Some calcium can also be taken up by the cells.

These findings are discussed briefly in connection with their bearing on problems of permeability to electrolytes of muscle cells.

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EFFECTS OF MARINE MUD UPON THE AEROBIC DECOMPOSITION OF PLANKTON MATERIALS [1]

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A number of recent investigations of bacterial activity in natural fresh and salt waters, in soils, and in muds strongly suggest that the rates of bacterial decomposition in these sites may be modified by finely divided and colloidal inorganic materials.

It is noted that marine bacteria are carried from suspension by settling marine and salt lake muds. To an undetermined degree encapsulated clumps, slime masses, and bacterial chains are mechanically occluded in the precipitating material, but the ratio of cells removed bears a relation to the electrical charges borne by the muds and bacteria characteristic of physical adsorption (3, 4, 5). Terrestrial soils also carry down adhering bacteria from suspensions. The degree of concentration may be varied by altering the charge on the soil particles (3).

Many marine and fresh water bacteria are sessile in habit, and it has been observed that the amount of bacterial substance in the attached films that these organisms form greatly exceeds the concentration in the surrounding water (6, 7). When the ratio of surface/volume in stored sea water is increased, the bacterial numbers rise and various physiological processes are stimulated. It has been suggested that the higher biological oxygen consumption associated with the increase in surface may be related to the adsorption and concentration of soluble organic materials and bacterial enzymes upon the enclosing surfaces (8).

Soluble and finely divided organic materials are themselves adsorbed on fresh water muds. River silts carry organic matter from muddy water to the bottom as they settle (2). From comparative studies of the biological oxygen demand characteristics, it appears that the organic matter in the silts breaks down at a much lower rate than that of the water from which they settled.

The experiments reported in this paper are attempts to discover if

[1] The author is indebted to the Elizabeth Thompson Science Fund for a grant of money which made possible the construction of the special respirometer apparatus and supplementary equipment used in this investigation.

[2] Contribution No. 257 from the Woods Hole Oceanographic Institution.

[3] Contribution from the Harvard Graduate School of Engineering No. 277.

marine muds affect the rates at which plankton materials undergo aerobic decomposition in the superficial layers of the sea bottom. Studies on the breakdown of sewage sludges indicate that aerobic conditions may prevail in the upper centimeter of the active mass, and it is probable that this also applies to the more flocculent surface muds receiving freshly settled debris from the sea (1).

Surveys of the distribution of organic matter and bacteria in marine muds indicate that biological changes go on relatively slowly in the sea bottom. It was thought, initially, that the adsorption of organic matter on the materials of the mud might be a significant factor in limiting bacterial activity.

Experiments on the Adsorption of Plankton Materials by Marine Mud

Materials

The mud used in these experiments was prepared from a series of short cores, 4 to 6 inches deep, taken from a station in the Gulf of Maine (N. 42° 25′, W. 70° 35′) at a depth of 100 meters. It had been stored for a year in the laboratory at room temperatures. A week before the work began the material was mixed with "aged" sea water and repeatedly suspended to remove heavy sand, fine gravel, shell particles, and other large fragments. A final suspension was made and aerated gently for three days. This was allowed to set—the heavy fraction separating within thirty seconds was discarded, and the remainder drawn off for use. The gross analysis of this mud was as follows:

Loss of weight on drying at 105° C. (corrected for salt content)	71.0 per cent
Loss of weight on ignition (on dry weight, salt-free basis)	11.5 per cent
Total nitrogen (on dry weight, salt-free basis)	0.20 per cent
Content of nitrogen per ml. wet mud as used in the experiments	0.70 mgm./ml.

Fresh plankton suspensions were prepared for each experiment from tows taken in Vineyard Sound (collected with No. 20 silk net, chilled, and prepared within four hours). This material consisted predominantly of species of *Rhizosolenia*. The tow was ground and filtered by suction through washed No. 52 Whatman filter paper. Nitrogen content of the extract was determined by micro-Kjeldahl, and convenient sea-water dilutions were prepared from the concentrated stock.

Determination of Adsorption

Varying concentrations of mud and plankton extract were stirred together on a gently rocking shaker for different intervals of time. Samples were withdrawn and nitrogen analyses made on aliquots of the mixture. Fifty-milliliter quantities of the mixture were placed in a clinical centrifuge and whirled until clear. Separate nitrogen determinations were made upon the mixture, upon the supernatant, and upon the centrifugate. The adsorption of plankton extract on mud in various

TABLE I

Adsorption of Nitrogenous Materials by Marine Mud

A. Plankton Extract

Concentration of N in extract 0.60 mg./ml.
Concentration of N in mud 0.70 mg./ml.

Ratio of mud to extract	N in supernatant after centrifuging (three-hour mixing)	N adsorbed by mud in three hours	N in supernatant after centrifuging (twelve-hour mixing)	N adsorbed by mud in twelve hours
	mg./ml.	*per cent*	*mg./ml.*	*per cent*
1 : 5	0.36	40	0.34	43
1 : 2.5			0.31	49
1 : 1	0.23	61	0.20	67
2 : 1	0.12	80	0.11	82

B. Peptone Solution

Concentration of N in solution 0.55 mg./ml.
Concentration of N in mud 0.70 mg./ml.

Ratio of mud to solution	N in supernatant after centrifuging (twelve-hour mixing)			N adsorbed by mud in twelve hours
	mg./ml.			*per cent*
1 : 25	0.42			23
1 : 2	0.13			77

mixtures under these conditions is demonstrated in the data given in Table I.

It is evident from this that the nitrogen-bearing fraction of plankton extracts are strongly adsorbed on marine mud. The concentrations involved in these experiments are several thousand times as high as those occurring in sea water but fall within the ranges that occur in marine muds.

Soluble materials such as plankton extract and peptone are readily

adsorbed at room temperatures, but bacterial digests of the same material are not removed to a comparable degree. In an experiment with a dense suspension of mixed bacteria grown in plankton extract, only 10 per cent of the nitrogen was adsorbed from suspension by mud stirred for 12 hours and centrifuged under conditions comparable to those applying in the other experiments. Microscopic examination of the mud thrown down showed the bacteria moving freely through spaces between particles—the centrifugate remained cloudy with motile bacteria.

Experiments on the Effect of Mud on Rates of Aerobic Decomposition

Breakdown of Particulate Plankton

Previous experiments on the rates of decomposition of organic matter in marine muds have been quantitatively unsatisfactory because of the technical limitations of the standard biological oxygen demand method. In these experiments the difficulties were eliminated by using a series of respirometers. The apparatus was designed by the author for the direct measurement of oxygen consumption of such organic matter rich systems as grossly polluted waters, sewage sludges, and manured soils. A description of their design and applications will be published in the near future.

In the first experiment the respirometers were filled with mixtures of mud and finely ground, unfiltered plankton. Duplicate respirometers were used for each mixture and filled as follows:

Set A—4 units mud in 200 ml. aged sea water
Set B—2 units mud, 1 unit plankton in 200 ml. aged sea water
Set C—1 unit mud, 2 units plankton in 200 ml. aged sea water
Set D—4 units plankton in 200 ml. aged sea water

Carbon analyses were not available for the plankton material, but satisfactory approximations may be made from the nitrogen determinations. Assuming a C/N ratio of 10:1 for the organic matter of the mud, the organic carbon value of the unit of mud is about 3.5 mgm. If a C/N ratio of 8:1 is taken for the plankton, the value of the plankton unit in terms of organic carbon is 2.4 mgm.

During the five-day term of this experiment the temperature was 23° C. $\pm$ 0.5° C.

The oxygen consumption of the various mixtures is plotted against time in Fig. 1.

Greatest irregularities occur in the duplicate samples of mud—possibly through the accidental inclusion of organic particles. It can be

assumed that the oxygen consumed in this interval was utilized in the biological oxidation of the organic carbon in the plankton material. On this basis a little over 50 per cent of the plankton carbon was oxidized during the five days of activity. The organic matter of the mud is more stable—in the same interval about 7 per cent of this carbon underwent oxidation.

In the mud and plankton mixtures the amounts of organic carbon oxidized to carbon dioxide are not significantly different at any time from the values that would be estimated from the consumptions of the separate

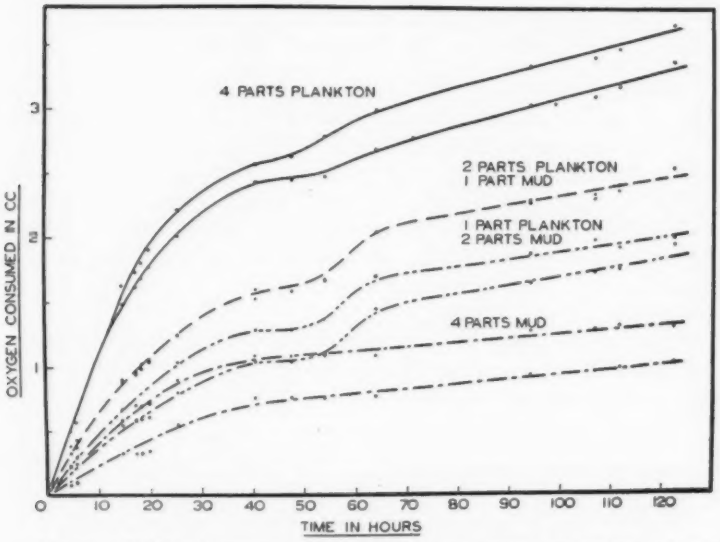

FIG. 1. Biological oxygen consumption in sea water suspensions of mud (lower set of curves), of ground plankton (upper curves), and of mixtures of mud and plankton (intermediate curves).

components. There is a slight increase in the proportion of carbon oxidized in the mixtures, but this effect is most pronounced in the lower concentration of plankton and may be related to the dilution effect observed in the third experiment.

Breakdown of Plankton Extract

A second experiment was prepared, using plankton extract instead of fine, particulate plankton. Mud, plankton-extract, and sea water were placed in sets of duplicate respirometers as follows:

Set A—4 units mud in 200 ml. aged sea water
Set B—2 units mud, 1 unit plankton, 200 ml. sea water
Set C—1 unit mud, 2 units plankton, 200 ml. sea water
Set D—4 units plankton in 200 ml. sea water

The nitrogen values of the mud and plankton units in the charges were adjusted to equal those of the first experiment. During the five days of activity the temperature ranged about 23.5° C. ± 0.5° C.

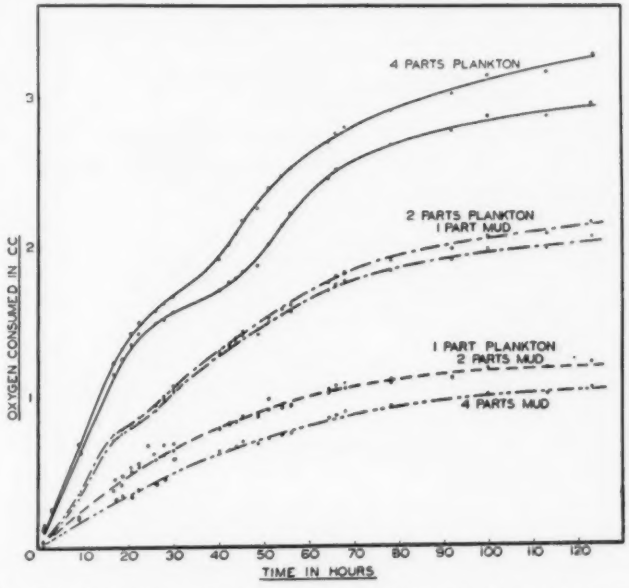

FIG. 2. Biological oxygen consumption of sea water suspensions of mud, plankton extract, and of mixtures of mud and plankton extract.

At the end of the second experiment approximately 45 per cent of the plankton carbon had been oxidized and between 6 and 7 per cent of the organic carbon in the mud. Very slightly more oxygen was consumed in the mixtures than would be calculated, but this difference is not significant when the greater efficiency of breakdown in the more dilute suspensions (Exp. 3) is considered.

Effect of Concentration on Decomposition in Mud

In the final experiment an attempt was made to discover what effect might be expected in rich mixtures of mud and plankton as compared

with the relatively dilute suspensions of mud and plankton used in the first two experiments. The respirometers were loaded in the following manner:

Set A—4 units mud (no added sea water)
Set B—2 units plankton extract, 2 units mud
Set C—2 units plankton extract, 2 units mud, 100 ml. aged sea water
Set D—4 units plankton extract (no added sea water)

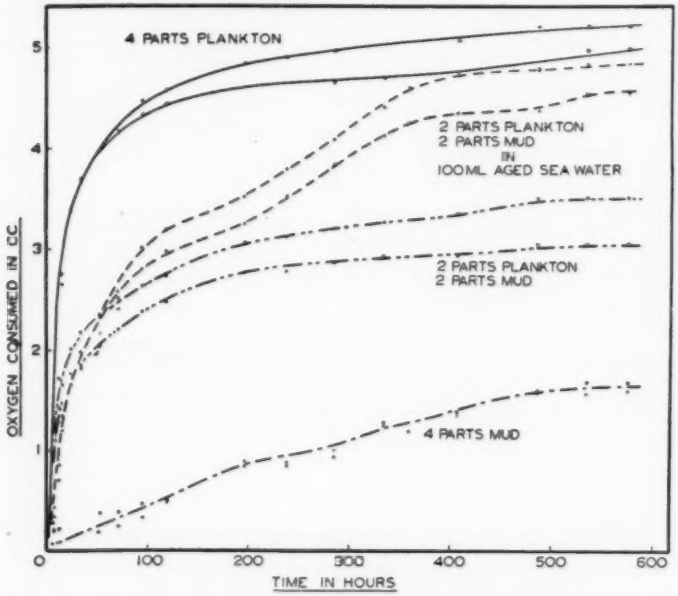

FIG. 3. Biological oxygen consumption concentrated mud, plankton extract, mixtures of plankton extract and mud, and of a sea water suspension of mud and plankton extract.

Although the rate of oxygen uptake had markedly decreased by the end of five days at 23.0° C. ± 0.5° C., the experiment was continued for 24 days.

At the end of this time 75 per cent of the organic carbon in the plankton extract had been oxidized and about 16 per cent of that in the mud. The undiluted mixture of mud and plankton consumed oxygen at a rate practically equal to that anticipated from the rates of consumption in the separate mud and plankton cultures.

The oxygen demand curve of the mud and plankton mixture diluted with 100 ml. of sea water is markedly different, however. It indicates a more rapid consumption of oxygen during the first hundred hours and a prolonged demand during the next three hundred hours. The oxygen consumed at the end of the active period—300 hours—is about 50 per cent greater than that taken up by the concentrated mud and plankton mixture. This effect of dilution is not related to the oxygen consumption of the added, aged sea water. Repeated determinations were made on the water alone and in no case did the oxygen demand of five-day intervals exceed 0.2 ml. per liter.

Discussion

It appears from these experiments that marine muds have little if any direct effect upon the rates at which readily decomposed organic materials that may be mixed with them undergo aerobic decomposition.

The fact that marine muds adsorb nitrogenous organic materials may be significant, however, in determining the normal course of their breakdown. Soluble organic matter released by autolytic or digestive processes may be localized in the mud where anaerobic changes and low temperatures prevail.

It is unlikely that silts perform transport functions in the sea as they do in the slow-flowing parts of silt-laden rivers, but by slowing down the rates of diffusion and maintaining relatively high local concentrations, muds may lower the effective rate of bacterial decomposition in the bottom.

Summary

1. Marine muds strongly adsorb soluble nitrogenous organic materials of plankton origin.

2. Muds exert little if any direct effect upon the rates or efficiency of anaerobic bacterial decomposition of plankton materials.

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THE LOCATION OF FACTORS OF HEAD REGENERATION IN THE EARTHWORM [1]

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Introduction

Common earthworms, within a certain limited extremely anterior region, can completely regenerate lost segments. At successively more posterior segments, the capacity for regeneration of characteristic head structures diminishes. Within a certain limited approximately middle region of the worm, a "tail" usually develops at a posterior cut surface, and a tail in reversed orientation (heteromorphic) may be produced at an anterior surface, but simple transection is never followed by development of a head. In the posterior region of the worm regeneration of a tail occurs at only the posterior cut surface.

These two questions arise. What determines regeneration? Is it a function of all of the organs and tissues present at the cut surface, or is some one part or group of parts responsible for the occurrence and character of regeneration? The discovery of "organizers" in embryonic development suggests the search for localized agencies determining regeneration.

A little analysis of the problem recognizes three major parts of the animal, in one or more of which the determining agencies may be seated: (1) the body wall, consisting mainly of epidermis (ectoderm) and muscle; (2) the digestive tube, including an internal epithelium and a muscle layer; (3) the central nervous organ. It seems unlikely that vascular organs or connective tissue should be directly concerned.

The central nervous structures have long been suspected of having some peculiar importance in relation to the occurrence and nature of regeneration. This has been investigated by Morgan (1902), Goldfarb (1909, 1914), Siegmund (1928), Avel (1932), Crowell (1937), and Bailey (1930, 1939). Very little has been said about the anterior body wall except that its epidermis may contribute to the regeneration of the new nervous parts. Among those who have taken a stand either for or against the epidermal origin of nervous parts are Hescheler (1898), Rand (1901), Nuzum and Rand (1924), Bailey (1930) and Schwartz

[1] Presented at the Thirty-sixth Annual Meeting of the American Society of Zoologists, 1938. *Anat. Rec.*, **72**: (4) Suppl., p. 85.

(1932). Morgan (1902), Hunt (1919), and Nuzum and Rand (1924) have dealt with the part played by the digestive tube.

The experiments described in the present paper were designed to test body wall, digestive tube, and nerve cord as to their relation to the initiation of regeneration and the kind of structure regenerated. The method consisted in transferring (by transplantation operations) one or two of the three parts in question from the anterior head-regenerating region to the more posterior tail-regenerating region, or in the reverse direction. The general idea was to make all possible combinations of body wall, gut, and nerve cord of head-forming and tail-forming regions of the worm.

Materials and Methods

The common earthworm, *Allolobophora caliginosa,* was used in all experiments. Among those used were a few (perhaps six) more highly pigmented than the others. Except for the pigment, however, no difference could be detected.

Previous to operation, the worm was anesthetized by placing in 0.2 per cent chloretone for about ten minutes. It was then laid on a glass plate covered by a wet paper towel. A simple tripod lens, or in difficult cases a binocular dissecting microscope, was used. Ordinary small scissors and forceps, sometimes jeweler's forceps, were adequate. Especially useful were sharpened needles inserted into large match sticks.

After the transplantation, stitches were taken with the use of a small needle (No. 12), and one strand of a fine three-strand silk thread. The worm was then placed in a glass tumbler between layers of wet paper toweling. The tumblers, each covered by a square of glass, were kept in a cold room at 3° C. for three or four days and then moved to a water-table where the temperature was about 7° C. This temperature was maintained for nearly three weeks. Following this, room temperature was considered most favorable for regeneration. At the desired stages, the worms were fixed in Bouin's picro-formol solution, serially cut in sagittal plane at 8 μ, and stained in Ehrlich's hematoxylin and eosin.

Experimental Work

Following is an outline of the several types of experiments employed:

Series A. Digestive Tube.

- Type 1. Gut and nerve cord of a region behind clitellum removed and pharynx implanted.
- Type 2. Gut and nerve cord of a region behind clitellum replaced respectively by pharynx and anterior nerve cord.

Type 3. Gut of a region behind clitellum replaced by pharynx; posterior nerve cord left in position.

Series B. Nerve Cord.

Type 1. Nerve cord of a region behind clitellum replaced by nerve cord from head-forming region.

Type 2. Nerve cord in head-forming region replaced by nerve cord from tail-forming region.

Series C. Body Wall.

Type 1. Segments from head-forming region, including gut but lacking the central nervous organs, sewed in normal antero-posterior orientation to anterior surface of a post-clitellar segment; protruding nerve cord of host component pulled into graft.

Type 2. Anterior body-wall rings from head-forming region slipped over intestine back of clitellum ("sleeve operation"):

(*a*) no central nervous organs present within graft;

(*b*) anterior nerve cord present within graft;

(*c*) posterior cord drawn forward into graft;

1. "sleeve" placed in normal orientation,
2. "sleeve" placed in reversed antero-posterior orientation.

The experiments in which rings of post-clitellar body wall were grafted over the pharynx are not included in the above outline. The operations were unsuccessful, due in large part to the stretching undergone by the posterior body-wall rings as they were pulled over the bulky pharynx. The single animal which survived the above operation gave no evidence of the initiation of either head or tail regeneration. A dozen other cases in which the pharynx was replaced by intestine from a post-clitellar region are omitted from the outline because none survived.

The post-clitellar region to which pharynx, anterior nerve cord, or anterior body wall was transferred lies at various levels between the fifth and twentieth segments behind the clitellum. From this same region parts were transferred to the anterior head-forming region of the worm. The work of Crowell (1937) showed that this post-clitellar region is capable of giving rise to a normal tail in a posterior direction, and a heteromorphic tail in an anterior direction. A series of controls set up by simple transection five, ten, and fifteen segments behind the clitellum verified Crowell's findings. This region was selected, therefore, because in normal regeneration it never produces anything except a tail.

The text figures illustrate not only the several types of experiments employed, but also particular cases which are to be considered as representative of the types. The plate figures are from photographs of sections, and should be compared with the diagrams of the results, shown in the text figures. In the case histories such expressions as "segments 4–11" are always to be understood as including the two segments whose numbers are given.

The text figures are diagrams of median longitudinal sections of the earthworm. The arrows indicate the source and new site of transferred parts and also the discarding of parts. The body wall is stippled; the nervous organs are in solid black. In the diagram representing the result of an experiment the wall of the digestive tube is cross-hatched. Omission of segments is designated by the irregular breaks in the diagrams.

Abbreviations:

A. N.c.	anterior nerve cord
C.	clitellum
I. N.c.	implanted nerve cord
Int.	intestine
P.-c.	post-clitellar
Ph.	pharynx
P. N.c.	posterior nerve cord
R. N.c.	regenerated nerve cord

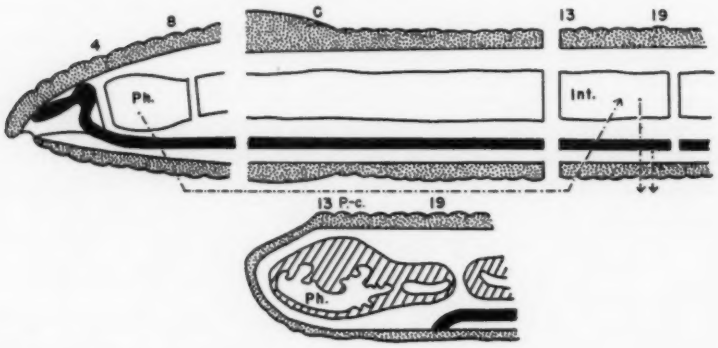

FIG. 1. The pharynx and oesophagus of segments 4–8 replace the intestine and nerve cord of post-clitellar segments 13–19. Result, shown below, is negative.

Series A. Digestive Tube

Type 1. Gut and nerve cord of a region behind clitellum removed and pharynx implanted.

Case 77 (*Figs. 1 and 12*).—The intestine and nerve cord of post-clitellar segments 13–19 were removed. To this position were transferred, in normal orientation, the pharynx and oesophagus of segments

4–8. The injured head region was then removed. After healing of the posterior incision through which the implant had been inserted, transection was made between the twelfth and thirteenth post-clitellar segments. The worm was fixed at the end of three and one-half months.

Although well oriented with reference to adjacent parts, the pharynx does not open to the outside (Figs. 1 and 12). The nerve cord, turned toward the ventral body wall, reveals little or no growth activity at its anterior end.

In most of the other cases of this type the transection following the healing of the longitudinal incision was made in such a way as to remove the anterior third of the pharynx. It was felt that establishing a freshly cut surface of the pharynx as well as of the body wall would be more conducive to regeneration. In all cases, however, the wound-surface healed over smoothly and no regeneration occurred.

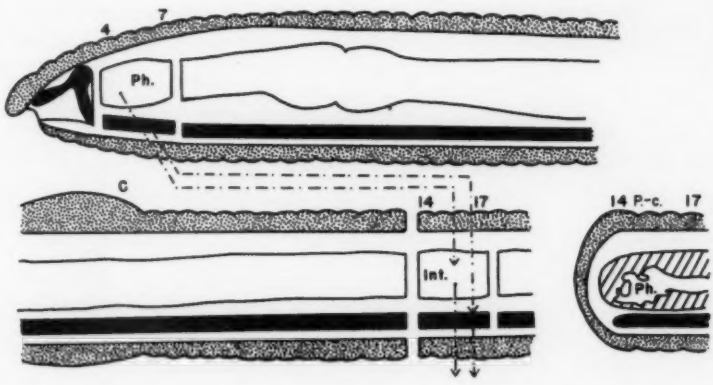

FIG. 2. The pharynx, oesophagus, and nerve cord of segments 4–7 replace the intestine and nerve cord of post-clitellar segments 14–17. Result, negative.

Type 2. Gut and nerve cord of a region behind clitellum replaced by pharynx and anterior nerve cord.

Case 11 (Figs. 2 and 22).—The intestine and nerve cord of post-clitellar segments 14–17 were removed. In their place were implanted, in normal orientation, the pharynx and a portion of the oesophagus together with the nerve cord from segments 4–7. After healing, transection was made at such a level as to remove a small part of the implanted pharynx. The remainder protruded to some extent. At the end of a month, the worm was fixed.

Except for a little proliferation at the forward end of the implanted cord, the result is negative (Figs. 2 and 22). Although communication between the oesophagus and the intestine was established, no anterior perforation to the outside occurred; the cut surface healed over smoothly.

An exceptional case (No. 84, Figs. 20 and 21) of this same type requires special attention. The implant, consisting of pharynx, oesophagus and nerve cord from segments 4–8, replaced the intestine and nerve cord of post-clitellar segments 13–23. After healing, transection was made immediately anterior to the implant. At the end of two and one-

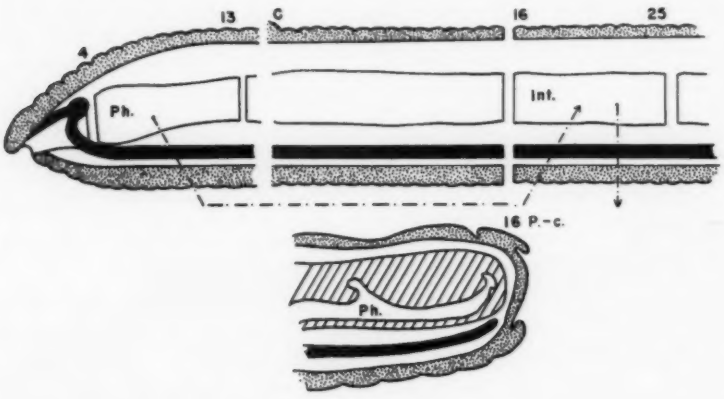

FIG. 3. With posterior nerve cord left in position, the intestine of post-clitellar segments 16–25 is replaced by pharynx and oesophagus of segments 4–13. Result, negative. The antero-posterior orientation of the lower part of the diagram is reversed to correspond to that of Fig. 16.

half months the worm was fixed. The sections (Figs. 20 and 21) show a well-formed brain, and commissures extending down to the nerve cord. Communication between the oesophagus and the intestine was established, but no anterior opening from the pharynx to the outside. The outstanding feature of the case is the close relationship between the nervous tissue and the pharynx epithelium (Fig. 21).

Type 3. Gut of a region behind clitellum replaced by pharynx; posterior nerve cord left in position.

Case 68 (Figs. 3 and 16).—The pharynx and oesophagus from segments 4–13 replaced, in normal orientation, the intestine of post-clitellar segments 16–25. The posterior nerve cord was left in position.

After healing of the longitudinal incision, transection was made between the fifteenth and sixteenth post-clitellar segments. Two and one-half months later the worm was fixed.

The sections reveal little cellular activity other than wound closure and healing. Certain sections show some proliferation of cells from the anterior end of the nerve cord, but give little evidence that a brain might have regenerated.

Series B. Nerve Cord

Type 1. Nerve cord of a region behind clitellum replaced by nerve cord from head-forming region.

Case 60 (*Figs. 4 and 23*).—The nerve cord of post-clitellar segments 10–17 was replaced by the anterior nerve cord from segments 4–11.

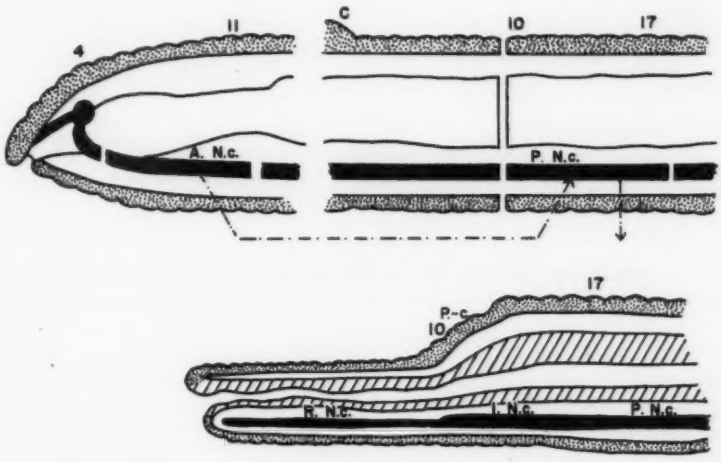

FIG. 4. Nerve cord of post-clitellar segments 10–17 is replaced by anterior nerve cord of segments 4–11. Result, heteromorphic tail. Implanted anterior cord has made connection with posterior cord.

The implant was made in normal orientation. After the healing of the posterior incision, simple transection was made just anterior to it, between post-clitellar segments 9 and 10. Examination at the end of two and one-half months reveals the presence of a heteromorphic

tail of about twenty-five segments. New nervous material extends to the tip of the regenerate. Because of the bending of the worm, no one section shows both the anus and portions of the new and the implanted nerve cord.

Type 2. Nerve cord in head-forming region replaced by nerve cord from tail-forming region.

Case 61 (*Figs. 5 and 13*).—A median longitudinal incision was made on the ventral surface of the thirteenth and fourteenth anterior segments. This permitted severing the cord between these two segments. Anterior to the fifth anterior segment the cord was grasped

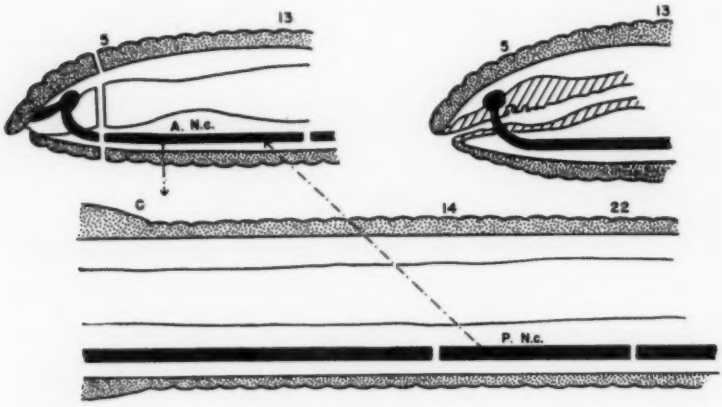

FIG. 5. Anterior nerve cord of segments 5–13 is replaced by nerve cord of post-clitellar segments 14–22. The first four segments are discarded. A new brain, commissures, three new segments, and a prostomium are regenerated.

firmly and pulled from segments 5–13. The nerve cord from post-clitellar segments 14–22 was pulled, in normal orientation, into these anterior segments by the use of silk thread and a long needle. The first four head segments, including the nervous organs, were discarded. At the end of two and one-half months the worm was fixed.

A new head with brain, commissures, and oral opening is evident. Three segments and a prostomium are new. The brain lies in the third new segment.

In Case 103, the removal of the anterior nerve cord from segments

6–12 was following by the implantation, *in reversed orientation,* of the nerve cord from post-clitellar segments 13–27. In two and one-half months a brain, lacking commissures, formed independently of the implanted cord. An anterior perforation of the gut to the outside did not occur.

Series C. Body Wall

Type 1. Segments from head-forming region, including gut but lacking the central nervous organs, sewed in normal antero-posterior orientation to anterior surface of a post-clitellar segment; protruding nerve cord of host component pulled into graft.

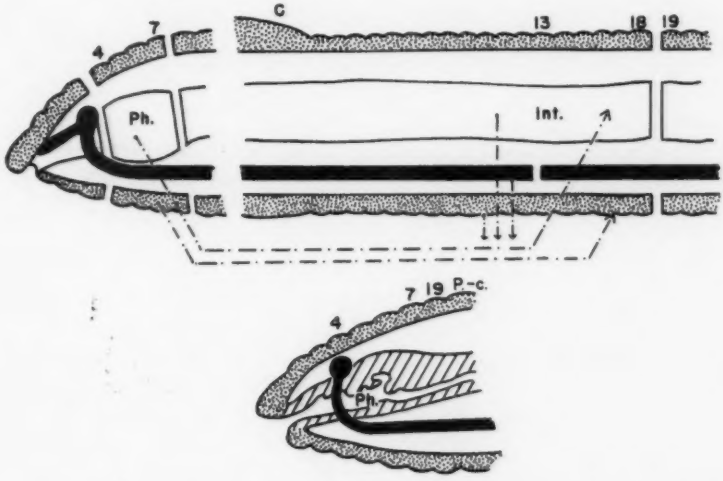

FIG. 6. Anterior segments 4–7, lacking the nervous parts, are grafted, in normal orientation, to anterior face of nineteenth segment behind the clitellum. The posterior nerve cord is drawn into the graft. Complete head regeneration results.

Case 47 (*Figs. 6 and 18*).—The anterior segments 4–7, including the digestive tube, but lacking the nerve cord, were sewed in normal orientation to the anterior face of the nineteenth post-clitellar segment. The nerve cord of post-clitellar segments 13–18 was pulled into the graft in the position occupied formerly by the anterior nerve cord. Two and one-half months later the worm was fixed.

Investigation reveals a regenerate possessing two segments, a prostomium, an oral aperture, a brain located in the third segment (first

of the graft or fourth of the original worm), and commissures extending down to the nerve cord. The pharynx communicates with the intestine by means of a short portion of oesophagus.

In this case occurs the same combination of parts as in Series B, Type 2. In both, the posterior nerve cord participates in head regeneration.

Type 2. Anterior body-wall rings from head-forming region slipped over intestine back of clitellum (" sleeve operation ");

(*a*) No central nervous organs present within transplant (" sleeve ").

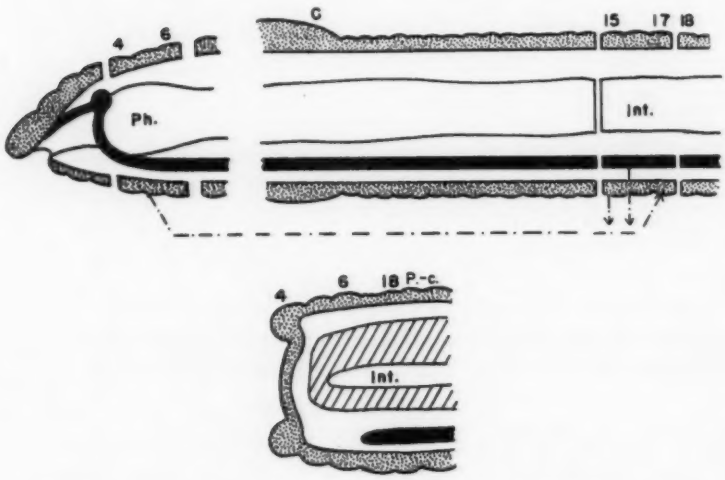

FIG. 7. A sleeve of anterior body-wall segments 4–6 is grafted over intestine of post-clitellar segments 15–17. The operated region contains no central nervous parts. Result negative.

Case 37 (*Figs. 7 and 15*).—A sleeve consisting of anterior body-wall segments 4–6 was slipped over the intestine of post-clitellar segments 15–17. All central nervous parts in the region of operation were discarded. The sleeve was normally oriented and sewed to the anterior face of the eighteenth post-clitellar segment. At the end of two months the worm was fixed. The sections show no activity except wound closure and healing.

In Case 17 the resulting conditions are somewhat different. The operation was the same as that above (Case 37) except that the sleeve was slipped over the intestine of post-clitellar segments 12–14 instead of 15–17. The length of time between operation and fixation was the same in both. In Case 17, however, a brain formed without any relation whatever to the ventral nerve cord. No commissures were evident; there is no anterior opening of the gut to the outside.

Cases 78 and 79 differ from both of the preceding in that the posterior nerve cord grew forward almost to the end of the sleeve.

Except these last three cases, all of this type agree in result with that of Case 37.

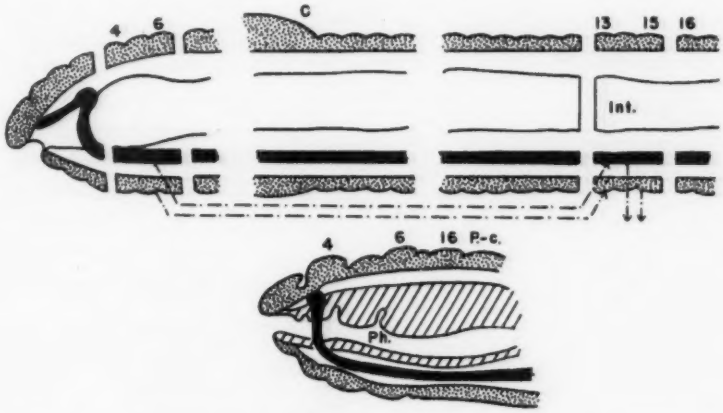

FIG. 8. A sleeve of anterior body-wall segments 4–6, including nerve cord, is grafted over intestine of post-clitellar segments 13–15. Head regeneration results.

(*b*) Anterior nerve cord present within graft.

Case 65 (*Figs. 8 and 14*).—After discarding the body wall and nerve cord of post-clitellar segments 13–15, a sleeve of anterior body-wall segments 4–6, together with their nerve cord, was slipped over the protruding intestine and attached (by stitches) in normal orientation to the anterior face of the sixteenth post-clitellar segment. At the end of two and one-half months the worm was fixed. Sections (Fig. 14) show a well-formed brain, commissures, typical pharynx epithelium, and an anterior opening of the gut to the outside.

Case 42 (Fig. 11) shows a regenerating brain and commissures, and an imperforate stomodeum. Figure 10, from another section of the same worm, shows the close relationship between the newly-formed brain and the epidermis.

(*c*) Posterior cord drawn forward into graft.

1. " Sleeve " placed in normal orientation.

Case 27 (*Figs. 9 and 19*).—A sleeve of anterior body-wall segments 4–6 was slipped, in normal orientation, over the intestine and nerve cord of post-clitellar segments 10–12. At the end of two months the

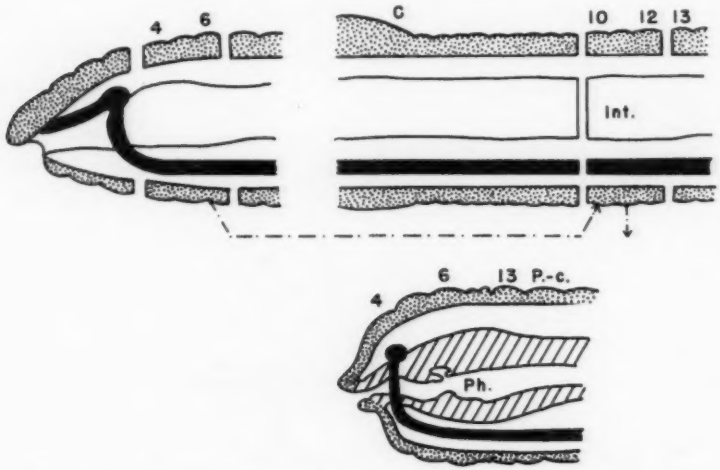

FIG. 9. A sleeve of anterior body-wall segments 4–6 is grafted over intestine and nerve cord of post-clitellar segments 10–12. Result, head regeneration.

worm was fixed. Sections show a new head with brain, commissures, a small amount of differentiated pharynx epithelium, and an oral opening.

With the exception of the oral opening, Case 32 possesses all the regenerated head parts noted in Case 27. The question of the origin of the pharynx epithelium will be discussed in another section of the paper.

Case 74 is another which clearly demonstrates the ability of the posterior nerve cord and intestine to participate in head formation when combined with anterior body wall.

The results of the three cases cited above, which agree essentially with those of seven additional cases, appear in Table I.

Three variations in the operative procedure, not included in Table I, showed different results. In Cases 75 and 83 the sleeve was oriented so that its dorsal side coincided with the ventral side of the host. As a

TABLE I

Explanation of Symbols

	Anterior	Posterior
Body Wall	W	w
Gut	G^p (pharynx)	g^i (intestine)
Nerve Cord	N	n

	Combination	Number of Cases	Result
Series A			
Type 1	wG^p	13	No regeneration
Type 2	wG^pN	8	No regeneration
		1	Head
Type 3	wG^pn	5	No regeneration
Series B			
Type 1	wg^iN	7	No regeneration
		1	Heteromorphic tail
Type 2	WG^pn	6	Head
Series C			
Type 1	WG^pn	5	Head
Type 2			
(a)	Wg^i	3	No regeneration
		1	Brain only
		2	Proliferation at end of nerve cord
(b)	Wg^iN	3	Head
(c)			
(1)	Wg^in normal orientation	10	Head
(2)	Wg^in W reversed antero-posteriorly	2	Results negative. Figure 17 shows slight regeneration.

result, the posterior nerve cord, pulled into the dorsal side of the sleeve at the time of operation, became attached dorsally by strands of connective tissue. No regeneration occurred.

In Case 44 the sleeve was oriented so that its dorsal side was lateral with reference to the dorsal side of the host component. At the end of a month and a half the posterior nerve cord had grown laterally to meet or give rise to new nerve material lying in the lateral plane (dorsal portion of sleeve).

2. "Sleeve" placed in reversed antero-posterior orientation.

Case 72 (*Fig. 17*).—A sleeve of anterior body-wall segments 4–6 was slipped over the intestine and nerve cord of post-clitellar segments 25–27. In contrast to previous cases, however, the sleeve was reversed so that segment 4 of the sleeve was sewed to the anterior face of post-clitellar segment 28. This left segment 6 of the graft in the anterior position. Normal dorso-ventral orientation of the graft was preserved. At the end of two and one-half months the worm was fixed.

Figure 17 shows a very slender anterior regenerate in the form of a finger-like projection. The nerve cord has grown forward and enters the base of the projection.

The results in this and in the one other case are the same; the activity of the nerve cord is confined entirely to the ventral side.

Out of more than four hundred worms used in the various experiments, only a small proportion survived. The significant results are summarized in Table I.

Discussion

The Rôle of the Digestive Tube in Regeneration

No experiment was performed with the express purpose of finding out whether or not regeneration would occur in the absence of the digestive tube at the wound region. The work of Morgan (1902) and Hunt (1919) shows that a head may begin to regenerate before the digestive tube, removed from the region just behind the plane of transection of the body, has grown forward to the wound region. In all the experiments performed in the present study some part of the digestive tube was present at the wound surface.

The pharynx alone, or in conjunction with the posterior nerve cord, failed to evoke head regeneration when implanted back of the clitellum. In like manner, the cases involving the combination of pharynx and anterior nerve cord in the posterior position are negative with the exception of Case 84. In this case, as already stated, there is a close relationship between the new nervous tissue and the pharynx epithelium (Figs. 20, 21). The characteristic columnar condition of the epithelium in this region has been lost. The layer, lacking a basement membrane, is made up of flattened cells with large, spherical nuclei. These epithelial cells merge uninterruptedly into the nerve cells so that there is no sharp demarcation between the epithelial layer and the brain anlage. Mitoses are abundant. There is very little evidence of proliferation

from the old nerve cord. The relatively great distance of the epidermis from the brain (Fig. 20), and the absence of mitoses in the epidermis preclude any idea of contribution to brain formation from this source. The similarity of the pharynx epithelial and the prospective brain cells, their close proximity, and the abundance of mitotic figures indicate that, in this case at least, the pharynx epithelium has contributed to brain formation.

The apparent manner of origin of the brain in Case 84 is very similar to that described by Nuzum and Rand (1924). As early as a week following removal of the brain, without removal of any other part, they found groups of deeply-staining cells lying just above and in close relation to the pharynx epithelium. According to them, "These cells, containing large nuclei with prominent nucleoli, are similar to the cells of both pharyngeal and epidermal epithelia. Their position, corresponding precisely to that occupied by the obvious brain fundament of later stages, as well as their character, marks them as the early brain fundament" (p. 218). Siegmund (1928) suggested that the pharynx may be concerned in the formation of a brain which arises independently of the nerve cord.

It is possible, then, that the pharynx, ectodermal in ontogeny, may have some head-determining capacity. Results obtained in Series C, Type 2, show that the intestine can participate in head regeneration (Figs. 11, 14, 19), and that the epithelium of the intestine can dedifferentiate and redifferentiate into pharynx epithelium. Case 32 gives evidence that the new pharynx epithelium is derived from the intestine since there is no oral perforation and hence no possibility of contribution to pharynx formation by stomodeal epithelium. The endodermal origin of pharynx epithelium in regeneration is described by Hescheler (1898, p. 581): "Das regenerierte Pharynxepithel geht aus den Zellen des alten Darmes hervor." Apparently, then, the pharynx is not essential to head regeneration even though it may, under some circumstances, be capable of giving rise to a brain.

The Rôle of the Nerve Cord in Regeneration

Morgan (1902, p. 571) maintained that the "cut-end of the nerve-cord is necessary at a cut-surface in order that a new head may develop." Bailey (1930) observed that regeneration does not occur when the nerve cord is looped back so that its cut end is remote from the wound. Results obtained by Goldfarb (1914), Siegmund (1928), Avel (1932), Crowell (1937) and Bailey (1939) show that the presence of the nerve at the wound surface is not essential to head regeneration; a brain may form

independently of primary connection with the nerve cord. In the present work two instances of this sort, described above, were obtained. In Case 17, the sleeve of anterior body wall, grafted over the intestine back of the clitellum, contained neither anterior nor posterior nerve cord, yet a new brain formed in the anterior segment of the sleeve. Its close relation to the epidermis and the similarity between the cells of the brain anlage and the flattened epidermal cells give evidence that this brain was derived from the epidermis of the transplant.

Case 103 consisted of the transplantation of posterior nerve cord, in reversed orientation, to the head-forming region. As in Case 17 above, a brain formed showing close relationship to the epidermis but having no cellular connection with the implanted cord.

In all such cases where a brain develops independently of cellular connection with the nerve cord, there is the possibility that the cord exerts an influence through some neurohumor-like substance not observable in histological studies.

Especially significant are the sleeve cases and the cord transplantation experiments which demonstrate that the posterior nerve cord, usually associated only with regeneration of normal or of heteromorphic tails, can participate in head regeneration, and that the anterior nerve cord from the head-forming region can participate in the formation of a heteromorphic tail. Nervous structures of new heads in whose regeneration either anterior or posterior cord has participated show evidence of cellular proliferation in the old nerve cord. It is evident, however, that the nerve cord does not determine the form of the regenerate, and so can not be of the nature of an organizer.

The importance of the part played by the brain cannot be denied. The establishment of a brain anlage is always the first event in head regeneration. If, however, the nerve cord is concerned in this establishment, the posterior nerve cord is just as effective as the anterior nerve cord. We must look elsewhere for a specific *head* determiner. As suggested by Crowell (1937), it is not improbable that the new brain, however determined itself, may act as organizer during the further course of head regeneration.

The Rôle of the Anterior Body Wall in Regeneration

According to Hescheler (1898, p. 580) the epidermis is the chief source of cells giving rise to regenerated nervous material. He reports: "Während der ganzen Dauer der Bildung des neuen Centralnervensystems war keine grössere zusammenhängende Abspaltung von Epider-

miszellen, sondern nur Zuwanderung einzelner Zellelemente zu beobachten." While Rand (1901) agrees with Hescheler that the epidermis is an important source of nervous material, Bailey (1930) observes no cells coming from the epidermis, and Schwartz (1932) claims that the connective tissue is the source of new cells in the cerebral ganglia. The observations made in the present study agree with those of Hescheler and Rand.

The rôle of the body wall is clearly seen in those cases where a sleeve of anterior body wall is grafted into the post-clitellar region in normal orientation with relation to the host component. A combination of such a sleeve, either anterior or posterior nerve cord, and intestine always evokes head regeneration. Attention has been called to Figure 17 (Case 72) which shows the negative results obtained when a sleeve of anterior body wall is grafted in reversed polarity over intestine and posterior nerve cord. This case, as well as the one other, involves the same combination of parts which, when all are in normal orientation, always produces a head.

Most of the normally oriented cases studied in this work represent such an advanced condition of regeneration that it is difficult to ascertain by what method the sleeve determines the form of the regenerate. Two cases, representing early stages in head regeneration, will be considered in more detail.

Case 17 was discussed briefly in connection with the rôle played by the nerve cord. The sleeve of anterior body-wall segments, enclosing the intestine back of the clitellum, contained neither anterior nor posterior nerve cord. The anterior end of the sleeve, at first open, closed in healing. The anterior cut end of the intestine likewise closed. Between the healed and closed anterior ends of body wall and intestine lies a cicatricial mass in which is embedded a brain anlage lying close to the epidermis. In the vicinity of the brain the epidermal layer, made up of flattened cells, is thinner than elsewhere. It is concave toward the brain, suggesting the beginning of a stomodeal invagination. The cells of the brain anlage, epidermis, and thin intervening mass are similar, all being of the epithelial type with large spherical nuclei and prominent nucleoli.

Case 42 (Figs. 10 and 11), in which an anterior body-wall sleeve and anterior nerve cord were grafted over the intestine, shows a developing brain, commissures, and an imperforate stomodeum. The relation between the brain and an inpocketing of the epidermis is seen in Fig. 10. Although ordinarily made up of columnar cells, the epidermal layer in this region consists of flattened cells and, furthermore, lacks the usual

basement membrane. The cells appear to be undergoing a process of redifferentiation, a process marked by active mitosis and a distinct migration of the newly-formed cells to constitute the regenerating brain. This migration gives, in places, the effect of "streaming" of cell masses. The evidence afforded by this and the preceding case makes it clear that the epidermis of the anterior body wall initiates head regeneration by contributing cells which go to make up the brain. The fact that head regeneration has never been observed to take place in the absence of a brain anlage indicates that the brain, once established, evokes further and complete development of the head.

The question may now be asked, in what region of the anterior body wall is located the seat of brain determination, the dorsal portion, the ventral, or the lateral portion? With the exception of number 84, in which the brain anlage bears a close relation to the pharynx epithelium, all cases in which brain formation has been described indicate that the dorsal portion of the body-wall sleeve is the site of determination. In cases 75 and 83 the sleeve, ordinarily dorso-ventrally oriented with reference to the host, was oriented so that its dorsal side coincided with the ventral side of the host. This resulted in the apposition of the end of the posterior nerve cord and ventral side of the intestine against the dorsal side of the sleeve. Although giving no evidence in favor of the dorsal side as the region of determination, the fact that no regeneration occurred suggests that the ventral side of the sleeve, placed in the dorsal position, plays no rôle in the initiation of brain formation.

In Case 44 the sleeve was oriented so that its dorsal side was lateral with reference to the dorso-ventral axis of the host. Sections reveal that the posterior nerve cord has grown forward and laterally from its ventral course to meet or give rise to new nerve material lying in the lateral plane (dorsal part of sleeve). If the new material comes partly or even entirely from the old cord, then apparently the direction of its regeneration has been determined by the misplaced dorsal side of the graft. If the new material has its origin in the dorsal region of the graft, it and the old cord have advanced toward each other and united. According to Joest (1897), if an earthworm be transected and the two parts grafted together so that they are not properly oriented, the ends of the nerve cord, if not too far apart, will grow toward each other and re-establish connection.

In conclusion, it may be said that, in head regeneration, the brain, once established by the epidermis of the dorsal body wall, takes over the direction of further head development including the establishment of commissures and their connection with the nerve cord. We may thus regard the dorsal epidermis of the anterior body wall as the primary head determiner, and ascribe to the brain the rôle of secondary organizer.

Summary

1. The presence of pharynx at a wound region is not essential to head regeneration even though it may, under some circumstances, be the source of nerve material (brain).

2. While obviously not a head determiner, the intestine can participate in head regeneration apparently as readily as the pharynx can. Intestinal epithelium can redifferentiate into characteristic pharynx epithelium.

3. The presence of nerve cord at a cut surface is not an essential factor in head regeneration since a brain may develop independently of any cellular connection with the cord. There may, however, be some hormonal relation between the nerve cord and regeneration.

4. The nerve cord does not determine the type of the regenerate for:

(*a*) The anterior (cephalic) nerve cord can participate in heteromorphic tail formation as well as in head formation.

(*b*) The post-clitellar cord (within the first twenty-five segments behind the clitellum) can participate in head formation as well as in normal and heteromorphic tail formation.

5. The "sleeve" operation, consisting in grafting over the intestine, in normal orientation, segments of anterior body wall containing either no central nervous parts, or anterior or posterior nerve cord, was invariably followed by head regeneration except in most of those cases in which no nerve cord was initially present. The "sleeve" cases afford strong evidence that the epidermis of the anterior body wall, being the source of the brain anlage, contains the primary determiner of head regeneration, and that the seat of this determination is probably located in the dorsal part of the epidermis.

Acknowledgment

It is a pleasure to express appreciation to Professor Herbert W. Rand, who suggested this problem and under whose helpful and critical supervision the work was performed.

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PLATE I

EXPLANATION OF FIGURES

Abbreviations used in plate figures:

Br. ..... brain
B.v. ..... blood vessel
I. N.c. ..... implanted anterior nerve cord
Int. ..... intestine
N.c. ..... nerve cord
Ph. ..... pharynx
R. N.c. ..... regenerated nerve cord

10. Case 42. High-power view of brain a few sections from that shown in Fig. 11 of the same case. Note proximity of brain to inpocketing epidermis. × 290.

11. Case 42. Regenerating head resulting from combination of a sleeve of anterior body-wall segments (4–6), anterior nerve cord, and intestine. Note imperforate stomodeum. × 29.

12. Case 77. Pharynx implanted thirteen segments behind the clitellum. No nerve cord within operated region. No regeneration. × 34.

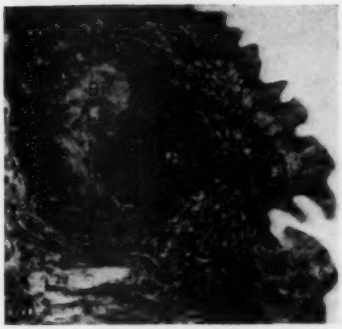
13. Case 61. Head regeneration occurred when posterior nerve cord replaced anterior cord. × 35.

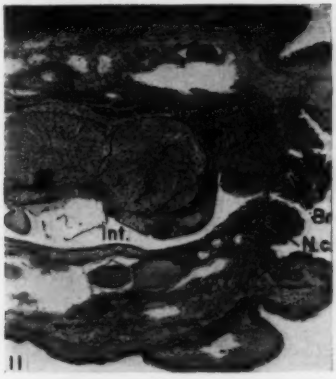
14. Case 65. Regeneration of a head following graft of sleeve of anterior body-wall segments (4–6) and anterior nerve cord over intestine. Other sections show stomodeal perforation complete. ×31.

15. Case 37. Sleeve of anterior body-wall segments (4–6) grafted over intestine. No nervous parts included. Regeneration did not occur. × 24.

16. Case 68. Pharynx implanted back of clitellum. Posterior nerve cord left in position. No head regeneration. × 36.

17. Case 72. Sleeve of anterior body-wall segments (4–6) grafted in reversed antero-posterior orientation over intestine and nerve cord back of clitellum. No head regeneration. × 33.





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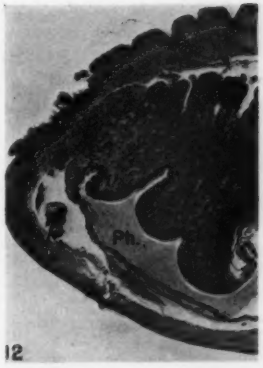

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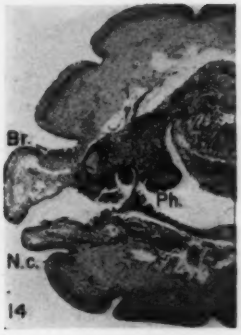

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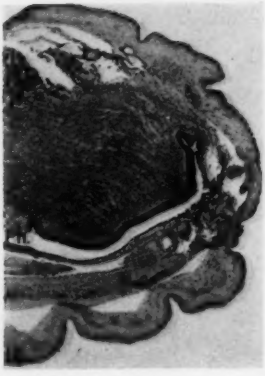

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PLATE I

PLATE II

Explanation of Figures

18. Case 47. Regeneration of head following graft of anterior segments (4–7) to posterior portion of worm. Posterior nerve cord drawn into graft, replacing anterior cord. × 33.

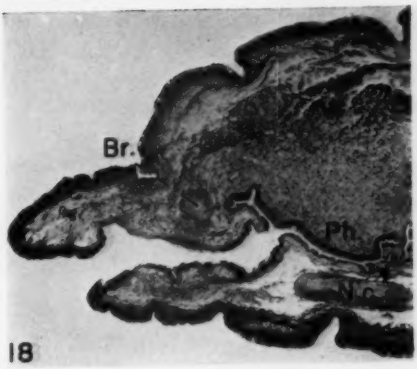
19. Case 27. Head regeneration following graft of sleeve of anterior body-wall segments (4–6) over intestine and nerve cord back of clitellum. × 45.

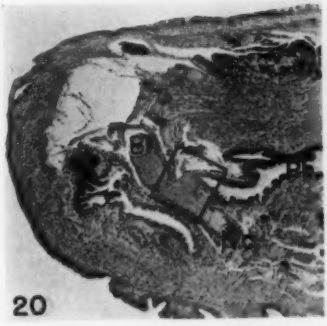
20. Case 84. Regeneration of a brain following implantation of pharynx and anterior nerve cord behind the clitellum. × 38.

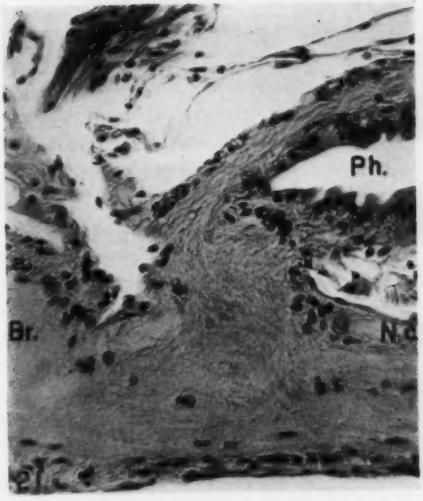
21. Case 84. Enlargement of that portion of Fig. 20 inclosed within square. Note flattening of cells of pharynx epithelium and the resemblance of its cells to the nerve cells. × 304.

22. Case 11. Pharynx and anterior nerve cord are implanted back of clitellum. Some forward growth of nerve cord but no brain anlage. × 42.

23. Case 60. A heteromorphic tail formed when anterior nerve cord replaced posterior cord back of clitellum. × 33.







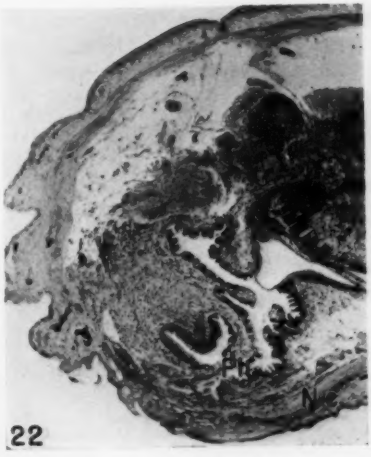


PLATE II

BEHAVIOR OF THE CELL SURFACE DURING CLEAVAGE

III. On the Formation of New Surface in the Eggs of Strongylocentrotus Pulcherrimus[1]

KATSUMA DAN AND JEAN CLARK DAN

(From the Misaki Marine Biological Station, Misaki, Kanagawa-ken, Japan)

In the preceding paper of this series (1938), it was shown that the surface of a sea urchin egg cleaving in Ca-free medium is stretched during the process of segmentation to cover the two resulting blastomeres, but that during the interkinetic period, after cleavage has been completed, this previously existing surface is reduced in area, and an apparently new surface is formed in the region involved in the cleavage furrow. This conclusion was drawn from numerous observations that adhering kaolin particles can be found at any point on the surfaces of the two blastomeres at the time when cleavage is completed, but that following this, the particles slowly retreat from the cleavage furrow region, and before the end of interkinesis this area is invariably quite devoid of particles.

Since this conclusion depended solely upon observations of particles attached to the outside of the denuded egg, it was obviously desirable to examine the cortical protoplasm, in order to rule out entirely the possibility that the particles were attached to some investing membrane whose behavior might be different from that of the true protoplasmic surface. An opportunity to settle this point was offered by the method of Motomura (1935), who reported observations on the behavior of pigment granules in the cortex of *Strongylocentrotus pulcherrimus* egg, which, he discovered, could be clearly seen with the aid of a blue filter. His conclusions, however, were considerably at variance with those of the authors, and it therefore became more interesting than ever to combine studies of the the particle-bearing surface layer with those of the granule-containing cortex. The results of this work are presented herewith in three sections. In the first will be presented a description of the pigment granules of *Strongylocentrotus pulcherrimus* eggs as well as some correlated observations on *Arbacia* eggs; the second part will deal with observations on the behavior of the *Strongylocentrotus* granules during cleavage and interkinesis; and the third, with combined observations of particle migration and granule behavior.

[1] This study was assisted in part by a grant from the Foundation for the Promotion of Scientific Research of Japan.

Pigment Granules of Strongylocentrotus, Arbacia pustulosa, and A. punctulata

For the original account of the pigment granules of *Strongylocentrotus pulcherrimus* eggs, Motomura's 1935 paper may be consulted. Briefly, they are small, orange in color, and poorly differentiated from the surrounding protoplasm when viewed with white or yellow light. If, however, a Wratten No. 49 filter is interposed between the eggs and the light source, the granules become more sharply differentiated, appearing dark-red or almost black with the light source used by the authors [2] (*see* Motomura, 1935, Fig. 64). In sections of eggs fixed by chrome-osmium mixtures or by chrome-formol fixatives with later osmication, the granules are strongly blackened.

The position and consequent visibility of these granules vary with the physiological condition and stage of cleavage of the eggs. For this reason, the results of three methods of observation—i.e., of living eggs with white and with filtered light, and of stained sections—have been combined to form the final picture of the distribution of the granules in unfertilized, fertilized and cleaved eggs.

Unfertilized Eggs in White Light.—Examination of the cortical region of living, unfertilized eggs under high magnification with artificial light or with sunlight (Plate I, *A*–1) shows structurally undifferentiated protoplasm of a light orange color extending to the "protoplasmic surface film" (*see* Chambers, 1938).[3] If the focus is raised, the surface of the egg appears to give off an orange color, but this color is diffuse and cannot be definitely attributed to any particular granules.

Unfertilized Eggs in Filtered Light.—If a Wratten No. 49 filter is introduced into the optical system, the egg acquires a just-perceptible, dark-reddish line at the circumference of the largest optical section, and when the focus is raised to bring the upper surface into view, scattered

[2] Osram point-light bulb mounted in a Leitz-Wetzler lamp with a transformer of 6 volts, 5 amperes.

[3] Before Chambers' paper came to their notice, the authors had performed various simple experiments to determine the real existence of this extremely thin, transparent layer which was so consistently found to be present that it was for some time thought to be a refraction artifact. It is visible with direct and dark-field illumination on eggs in sea water and Ca-free medium, and on exovates formed by compressing either fertilized or unfertilized eggs in sea water. On eggs normally fertilized in sea water, the protoplasmic surface film can be distinguished lying beneath the hyaline plasma layer. If such eggs are transferred to Ca-free medium, the hyaline plasma layer dissolves away inside the fertilization membrane, leaving the underlying surface film as the only visible structure closely investing the egg. In only one case was it found possible to effect a change in the appearance of this film. When unfertilized eggs were compressed, and then released before they burst, the protoplasmic surface film on the contracted surface lost its usual perfect contour, appearing roughened and irregular.

granules of this color appear, as nearly as can be determined, immediately below the protoplasmic surface film (Plate I, *B*–1).

Unfertilized Eggs in Section.—Unfertilized eggs treated with OsO_4 show a layer of blackened granules, which are distinguished from other granular inclusions by their slightly larger size, and lie so closely beneath the protoplasmic surface (Plate I, *C*–1) that they appear to be in contact with it.

Fertilized Eggs in White Light.—In less than two minutes after fertilization or after artificial activation, the egg in optical section presents a quite different picture, aside from the appearance of the fertilization membrane and the gradually thickening hyaline plasma layer. In white light, beneath the protoplasmic surface film, a zone of extremely finely

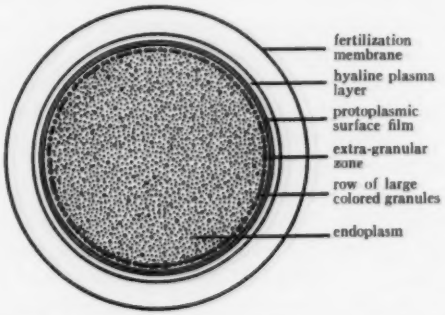


FIG. 1. Diagram of fertilized egg of *Strongylocentrotus pulcherrimus* in sea water.

granular (but not hyaline) protoplasm has appeared, apparently as a result of the centripetal migration, by approximately 1.5 μ, of the coarse granules which had been occupying this region (Fig. 1 and Plate I, *A*–2, *A*–3). This zone, which will hereafter be called the "extra-granular zone," gradually widens, reaching a width of about 2 μ before cleavage begins. Centripetal to the extra-granular zone and forming an outer boundary to the central heterogeneous granular protoplasm is a slightly irregular ring of larger, definitely orange granules, whose color is transmitted to the adjacent extra-granular zone. If the focus is raised, it can be seen that the upper surface no longer gives off the orange radiation which appeared in the unfertilized egg.

Fertilized Eggs in Filtered Light.—When observed with filtered light, the fertilized egg in optical section is seen to have acquired a prominent ring of dark-reddish granules whose position corresponds to that of the orange granules seen with white light (Plate I, *B*–2). At the surface

level, these present the same picture as those in the unfertilized egg, i.e., they lie scattered at random.

Fertilized Eggs in Section.—Osmicated fertilized egg sections show a row of strongly blackened granules at a corresponding distance within the egg cortex [4] (Plate I, *C*–2). On the basis of these observations and others presented below the authors are convinced that the granules which appear as the prominent ring in the fertilized egg move *inward* at fertilization from their previous position immediately inside the protoplasmic film, rather than *outward* from the endoplasm. The fact that their presence in the egg before fertilization can be detected only as a diffuse orange radiation can be explained on the ground that they are lying so closely in contact with the concave surface of the outer protoplasmic surface film as to be individually indistinguishable in optical section.

The conspicuous red granules of the *Arbacia* egg have been objects of interest for many years (Harvey, 1910; McClendon, 1910, et al.). It has generally been accepted that these granules move from the interior of the egg to the cortex at the time of fertilization, but this observation is difficult to prove. However, it is certain that a demonstrable rearrangement takes place during the four and a half minutes following fertilization in that the granules, which in the unfertilized egg appear to be located at various depths from the surface, move into a single layer in the now firmly gelated cortex (Plate II, *B*, *C*). This behavior is observed in the eggs of both *Arbacia punctulata* of Woods Hole and *A. pustulosa* of Naples. Although the details of the *Arbacia* granule movement are decidedly at variance with those which have been observed in *Strongylocentrotus*, the possibility seems to be worth considering that the rearrangement may be caused by inward migration, as is the case in *Strongylocentrotus*, rather than by the outward movement generally regarded as the sole process involved. This same change in distribution occurs after artificial activation.

Pigment which can be extracted from the tests of sea urchins is generally known as "echinochrome" after MacMunn's nomenclature, and it has been proved that the pigment is an oxidation-reduction dye (MacMunn, 1885, 1889; Griffiths, 1892; Cannan, 1927). Pigment which is contained in the eggs of several kinds of sea urchins including *Arbacia* ("arbacine" of Vlès and Vellinger, 1928) has also been identified as echinochrome (McClendon, 1912; Ball, 1934), and the observation that the granules move to the cell periphery after fertilization concurrently with a great increase in respiration lent further support to the

[4] Because of the shrinkage of the eggs incident to fixing and sectioning as well as the difficulty of determining the exact level of section in a given case, and since the distances in question are very small, the making of precise measurement was rejected as involving too large an error to have any meaning.

conclusion that they perform a respiratory function (Runnström, 1928). Crude trials were made to determine whether observable changes take place either in the orange coloration or in the spectroscopic absorption

PLATE I

A. Strongylocentrotus eggs in white light.

A–1. Unfertilized egg. Notice that there is no differentiated structure inside the egg.

A–2. Fertilized egg in Ca-free artificial sea water. Fertilization membrane has been removed mechanically and hyaline plasma layer has dissolved away. Note extra-granular zone around periphery and row of granules inside it. Dark appearance of granules is due rather to high refractivity than to intense color. Protoplasmic surface film is not clearly defined in this photograph.

A–3. Fertilized egg in normal sea water, showing fertilization membrane, hyaline plasma layer, protoplasmic surface film, extra-granular zone and row of granules (cf. diagram, Fig. 1).

B. Strongylocentrotus eggs in filtered light—mixed culture of fertilized and unfertilized eggs in Ca-free medium. (Since sea-urchin eggs cannot be fertilized in Ca-free medium, there was no danger of contamination of unfertilized eggs by excess sperm from fertilized eggs. As extra precaution, eggs were well washed after fertilization and allowed to stand some time before being mixed with unfertilized eggs.) In order to insure easy differentiation between fertilized and unfertilized eggs, fertilization membranes were not removed, but were left in place in collapsed condition which results upon their introduction into Ca-free medium. This collapse of fertilization membrane permitted simultaneous focusing upon fertilized and unfertilized eggs, which is impossible when membranes are normally expanded. Aside from collapse of membrane and disappearance of hyaline plasma layer, appearance of eggs in sea water and Ca-free medium is identical.

B–1. Unfertilized egg, showing absence of differentiation in granular cytoplasm.

B–2. Fertilized egg, showing dark-red ring. (Outermost structure is collapsed fertilization membrane, beneath which protoplasmic surface film is poorly visible at right side. Hyaline plasma layer has dissolved away in Ca-free medium.)

C. Strongylocentrotus eggs in sections.

C–1. Unfertilized egg showing row of osmium-blackened granules closely applied to cell surface.

C–2. Fertilized egg with extra-granular zone and row of granules beneath it.

D. Strongylocentrotus egg in late interkinesis—Ca-free preparation showing difference between "old" surface on polar side of blastomeres provided with extra-granular zone, and new surface along cleavage furrow without extra-granular zone. Note high refractivity of newly-formed surface.

E. Strongylocentrotus egg in four-cell stage (Ca-free medium). Note that "old" surface appears only on outer sides of blastomeres.

F. Strongylocentrotus egg in section. Separated blastomere in which polar and furrow regions are clearly differentiated by contour, showing absence of extra-granular zone and presence of highly refractive membrane in furrow region.

G. Section of spherical separated blastomere of *Strongylocentrotus* in which direction of preceding cleavage is indicated by differentiated structures as in *F*.

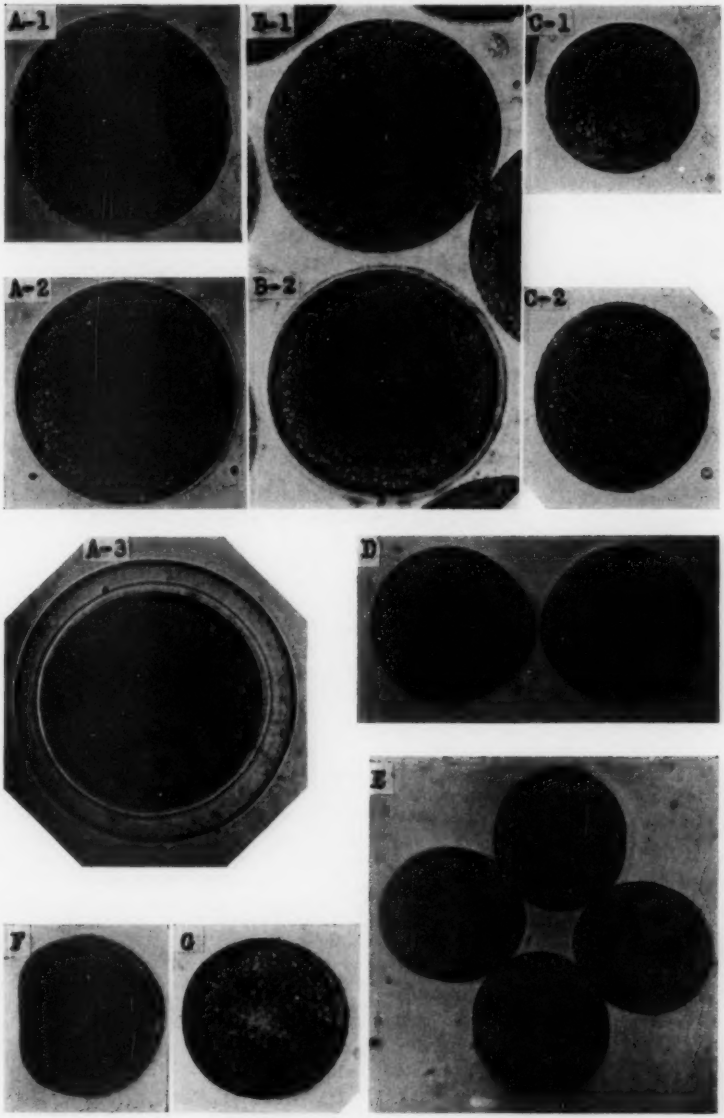


PLATE I

of the *Strongylocentrotus* granules under the influence of oxidants or reductants. Although it is still premature to make a final conclusion, at least in water extract and acetone extract of the pigment and in the pigment *in vivo*, no change could so far be detected, contrary to the case of the pigment extracted from the tests of this species (Suto, 1938).

A further marked difference in physiological function between these granules and those of *Arbacia* is found in the fact that these granules do not break down upon cytolysis or liberation into sea water, while the *Arbacia* granules do (Heilbrunn, 1928). However, the somewhat similar position and the fact that there is a remarkable change in distribution following fertilization suggest that there may be a closer relationship between them than is apparent from the existing data.

Behavior of the Cortical Elements in Cleavage

In this section, because of its importance in the later discussion, the behavior of the cortical elements in eggs cleaving in Ca-free artificial sea water will be described in detail.

The eggs of *Strongylocentrotus pulcherrimus* were prepared for observation by the following procedure: insemination was performed in a minimum of sea water, and when the fertilization membranes were par-

PLATE II

A. Consecutive photographs of division of *Strongylocentrotus* egg taken with direct sunlight through filter at low magnification, later enlarged. This series aims especially to show interrelation of dark-red ring and kaolin particles. (Room temperature 13° C., process plates used for photographing.)

A–1. Two kaolin particles stuck on sides of furrow (indicated by arrows). Note that dark-red ring completely covers surface.

A–2. One minute later. Furrow has deepened and particles are carried further into it.

A–3. Two minutes, thirty seconds later (than first photograph). Cleavage is complete. Note that dark granules are accumulating around point of last connection between blastomeres.

A–4. Three minutes, thirty seconds later. Blastomeres have rounded up.

A–5. Five minutes later. Dispersion of granules and migration of kaolin particles out of furrow have started simultaneously.

A–6. Six minutes later. First sign of opening of granular ring appears.

A–7. Seven minutes later.

A–8. Ten minutes later. New surface has fully developed. Note that kaolin particles are well outside furrow and dark granules are distributed only on polar sides of blastomeres.

B. *Arbacia pustulosa*, unfertilized egg in dark-field illumination. Surface view of egg, showing that pigment granules are at different depths from surface, so that simultaneous clear focus is not obtainable. (Note that this is reverse of case with *Strongylocentrotus*, in which granules are in single layer closely applied to protoplasmic surface film.)

C. Surface view of fertilized egg of *A. pustulosa;* pigment granules are now in a single layer.

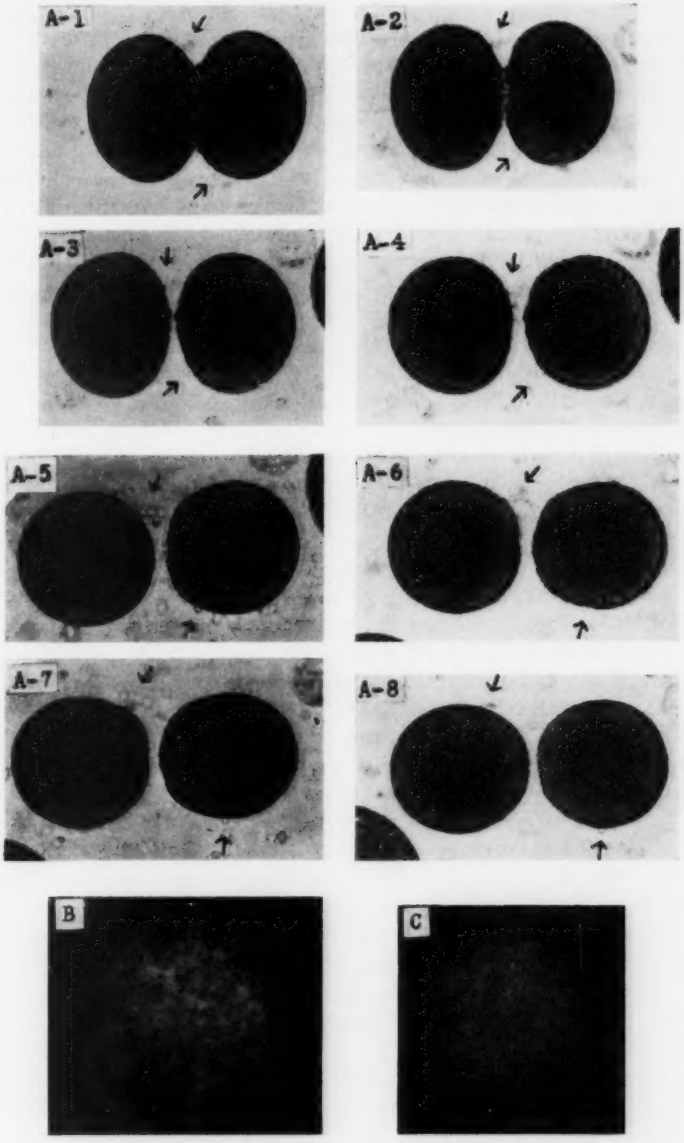


PLATE II

tially raised (about 1 minute later), a mixture of equal parts of Ca-free artificial sea water and 0.53M (isotonic) KCl was added to the eggs. In this solution, the membranes became abnormally expanded and fragile, and were removed without injury to most of the eggs by the use of a fine pipette of the proper bore. Since too long a stay in the presence of an excess of KCl produces abnormalities in the eggs at the time of cleavage, they were returned to Ca-free sea water, washed repeatedly, and allowed to develop until just before the first cleavage. At this time, an equal amount of isotonic KCl was again added to the suspension (since it was found that, in this species, in Ca-free sea water alone the blastomeres tend to come into close contact during interkinesis, rendering observation difficult, while this does not occur if excess KCl is present, although in all other respects the cleavage picture is the same.)

In White Light.—As cleavage begins in *Strongylocentrotus* eggs, the mutual relations of the granular ring, the extra-granular zone and the protoplasmic surface film are maintained practically unchanged. However, as the furrow progressively deepens, the extra-granular zone at the polar regions [5] becomes noticeably narrower, presumably because of stretching (Fig. 2, *A, B, C*). About the time when the separation of the blastomeres is complete, the granules appear to accumulate to some extent in the cortical protoplasm forming the walls of the furrow, and, moreover, are not so uniformly equidistant from the surface in this region (Fig. 2, *D*). During the first part of the interkinetic period, the accumulation of granules in the walls of the furrows is gradually dispersed, and the extra-granular zone in the furrow region, which has become continuous by the complete severance of the blastomeres, is gradually opened, starting from this central point (Fig. 2, *D, E*). As it opens, its place is occupied by an increasing extent of surface which is remarkably similar to that of the unfertilized egg; i.e., no extra-granular zone is present, the orange granules are not separately distinguishable and the coarse granular endoplasm extends all the way to the surface film (Fig. 2, *E, F*). However, this outer membrane is sharply defined against the surrounding medium because of a high refractive index which is found only in this newly-formed surface, and is not characteristic of the polar surfaces of the blastomeres (Plate I, *D*) or of the unfertilized egg surface (Plate I, *A*–1).

In the second cleavage, a practically similar process is repeated, the formation of new surface taking place in such a way that the extra-granular zone comes to lie only on the outer surfaces of the four blasto-

[5] In the papers of this series, the authors have adopted the terminology of Chambers with respect to the topography of cleaving eggs; i.e., the furrow is referred to as the equator in defining the poles. (See diagrams in previous papers.)

meres (Plate I, *E*); and so far as the authors can determine, this is regularly kept up until the blastula stage (see Motomura, 1935, Fig. 71).

In Filtered Light.—In the cleaving egg seen with filtered light, the dark-red ring covers the entire surface (Plate II, *A*–1, *A*–2). When

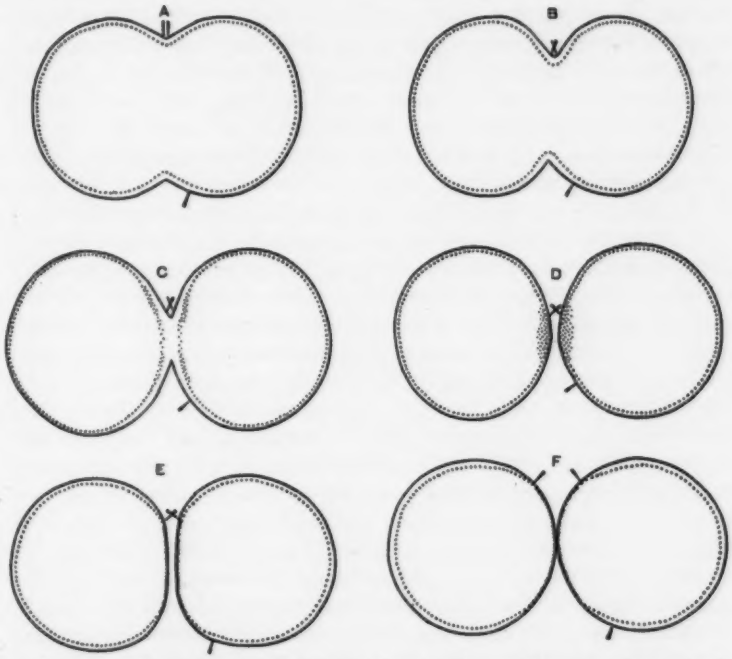


FIG. 2. Diagram of typical *Strongylocentrotus* experiment to show, simultaneously, behavior of kaolin particles adhering to surface, extra-granular zone and granular layer. (Changing outline of cleaving egg and positions of adhering particles are traced from photographs. Positions of granules are indicated by dots.)

A. Cleaving egg; kaolin particles are situated in furrow region; extra-granular zone has narrowed at poles, presumably because of stretching.

B. Kaolin particles are carried inward by deepening of furrow.

C. Extra-granular zone in furrow region widens, at poles becomes extremely narrow.

D. Extra-granular zone regains uniform width around blastomeres; granules are accumulated at point of last connection between blastomeres.

E. Formation in furrow region of new surface, lacking in extra-granular zone. Kaolin particles have been pushed out of furrow; accumulated granules have dispersed—they are now lined up in close proximity to newly-formed surface.

F. Late interkinetic stage; extra-granular zone over polar two-thirds of blastomeres has widened as though released from tension; upper particles are now well outside of furrow, at boundary between old and new surfaces.

the severance of the blastomeres is nearly completed, the dark-red ring in optical section becomes very thick at the sides of the furrow (Plate II, *A*–3, *A*–4); after cleavage is completed, it first regains a more uniform width all around the blastomeres (Plate II, *A*–5) and then breaks at the hyaline connecting stalk (Plate II, *A*–6, *A*–7) and becomes less and less a complete ring, finally including only about 65 per cent of the circumference of the blastomeres (Plate II, *A*–8). The surface where the ring has opened is characterized by the absence of the red granular ring, as is the case with unfertilized eggs. In the four-cell stage, the dark-red granules are distributed on the outer sides of the four blastomeres, corresponding exactly to the distribution of the extra-granular zone as seen in white light.

Sections.—In sections of eggs fixed with osmium mixtures, however, contrary to the expectation from observations in filtered light, there is a row of blackened granules extending completely around both the newly separated blastomeres and blastomeres in the interkinetic period. In the early stages of cleavage, at the poles, the ring is closer to the surface —i.e., the extra-granular zone is narrower than in the other regions; while in the cortex adjoining the furrow, the granules are less perfectly aligned, sometimes lying 5 or 6 μ inside the surface layer. In the later interkinetic stages, the ring of granules extends around the polar part of the blastomere section at a uniform distance from the surface as in the egg before cleavage. Around the periphery on the furrow side, this separating zone of protoplasm (extra-granular zone) is quite lacking, and the row of blackened granules is found closely applied to the surface membrane, as in unfertilized eggs. However, in this case, the granules appear to be more numerous, forming a continuous, highly refractive line (Plate I, *F*, *G*). This greater accumulation of granules in fertilized eggs than in unfertilized eggs may explain why this part of the blastomere surface is more clearly defined, in the living condition, than that of the unfertilized eggs.

When fertilized eggs are allowed to cleave in sea water, so that the fertilization membrane and hyaline plasma layer are intact and the blastomeres closely in contact as soon as the cleavage furrow is complete, it is impossible to distinguish either orange granules or dark-red ring along the contact surface (Motomura, Fig. 70). However, studies of sections reveal that, as is the case when cleavage takes place in Ca-free sea water, the extra-granular zone covers the entire surface before the complete severance of the two blastomeres, but that later it retreats from the furrow region while the row of granules is always encircling the endoplasm. From this, it must be concluded that the apparent absence of orange color in white light and of the dark-red ring in filtered light is

controlled not by the actual absence of the granules but rather by the optical conditions imposed by the presence of the extra-granular zone or by the spatial relations of the blastomeres.

Another indication that the apparent absence of the dark-red granules in the walls of the cleavage furrow in sea water is an optical illusion arising from the very close association of the two surfaces is furnished by the observation that when two fertilized eggs with perfectly complete rings of dark-red granules are brought into contact, the granules appear to be absent from the parts of the egg peripheries which are in contact. Therefore, since the visibility of the dark-red ring with a filter appears to be largely dependent upon the optical conditions, it must be concluded that one cannot safely determine the presence or absence of granules on the sole basis of observations with the filter.

Summarizing this section, it can be said that direct observation of the cortex reveals, as was predicted in the previous paper from the behavior of the kaolin particles, that the existing surface of the uncleaved egg is pulled into the furrow during the process of segmentation, and that only when the interkinetic period is reached, does a new surface begin to be formed along the furrow sides.

Simultaneous Observation of Cortical Elements and Kaolin Particles Affixed to the Surface of Strongylocentrotus Eggs

Kaolin particles were added to a Ca-free culture of the eggs, prepared in the way described above. After thorough mixing, excess particles were removed by washing. Eggs were mounted in a deep hollow slide, a cover glass was fastened in place by means of vaseline, and the whole preparation was immersed in a larger volume of Ca-free medium to reduce local heating by the light source.

When photographs were taken with filtered light, in order to make the exposure time as short as possible, direct sunlight and a low magnification were used. An egg with suitably located particles was selected and photographed at intervals from before the completion of cleavage until nearly the end of the interkinetic period. Figures shown in Plate II are enlarged pictures of such a series. In these photographs, a clear image of the extra-granular zone was sacrificed in order to show the dark-red ring.

In these series, eggs were chosen with particles at the head of the cleavage furrow, because this is the spot where it is anticipated that the new surface will begin to appear. If so, the particles which are at the head of the furrow are expected to come to lie at the border between the old and new surfaces when the latter is formed. In filtered light, this border will be the place where, in optical section, the dark-red ends. In

white light, such particles will be found where the extra-granular zone ends. This expectation has been borne out perfectly by many observations.

The unfailing coincidence between the position of such kaolin particles and the border line between the old and new surfaces (*see* diagram, Fig. 2, *E, F*) leads to the conclusion that the behavior of the kaolin particles can be taken as a direct indication of the behavior of the cortical material. Evidently, the kaolin particles must be adhering to the protoplasmic surface film, which is the outermost structure persisting in a Ca-free medium, and the above findings show that the protoplasmic surface film does not slip over the underlying cortical layer as does the hyaline plasma layer [6] (Dan, Yanagita and Sugiyama, 1937).

Discussion

In the preceding sections the observations have been presented in the light of the authors' interpretations. However, since Motomura's interpretations sometimes differ from those of the authors, a few words will be devoted to comparing the two.

In connection with cleaved eggs in sea water, Motomura concluded that the granules are present only along the free surfaces of the blastomeres and that they are absent along the contact surfaces,—a conclusion based on the fact that the dark-red granules in filtered light can be seen in the former position but not in the latter. So far as the observation is concerned, our finding coincides perfectly with Motomura's. However, if the identity of the dark-red granules and the osmium-stained peripheral granules in sections is assumed, as the authors are compelled to do from the coincidence in size, position and behavior between them, it must be concluded that the granules are present all over the surface at any time, and that it is the extra-granular zone which is present only on the free surfaces and not along the newly-formed surfaces (Motomura, Fig. 70). The authors' explanation of the observation is that the granules will take a dark-red color in filtered light anywhere and at any time, but when they are closely in contact with a surface, it is not possible to distinguish them. However, both sets of conclusions agree in saying that the part of the surface of cleaved eggs in which the dark-red granules are invisible in filtered light is a newly-formed surface.

Concerning the case in which eggs are kept in a Ca-free medium and are allowed to cleave in it, Motomura came to a rather confusing conclu-

[6] It incidentally follows that a new protoplasmic film must be produced over the newly formed surface of the blastomeres. This is to be expected from the previously reported observations, in which no intact protoplasmic surface could be found without the investing film.

sion. Quoting him: "In short, the new cell boundaries are not formed in the calcium-free sea water in the course of the early cleavages. The surface of each blastomere is covered by the extension of the pigmented cortical cytoplasm. But, when the blastomere is replaced in the normal sea water, it regains the capacity of forming the new cell boundaries. In this case the blastomere is able to form the pluteus. When the egg is cultured successively in calcium-free sea water, no formation of the embryo in it is possible. In this case the distribution of the pigment granules is irregular" (p. 239). From this description, he apparently missed the formation of new surface in Ca-free sea water entirely. In the authors' experience, in order to observe the formation of new surface, special precautions are necessary to secure a good condition of the cultures. Overheating of the preparation by the light source or a slight shortage in the quantity of the culture medium at the time of observation immediately impairs the eggs. During the first cleavage, when such injury occurs, the eggs invariably stop their development at the stage in which the separation of the blastomeres is just completed and the granules are accumulated at the sides of the furrow (Plate II, *A*–3, *A*–4). Therefore, even as early as the beginning of the interkinetic period, it is possible to predict whether or not an egg under observation will undergo further cleavages. Cultures were encountered, especially toward the end of the season, in which all the eggs remained in this state. In such eggs, the granules sooner or later begin to clump together irregularly, and the eggs finally die. But so far as the authors are aware, as long as the condition of the egg remains favorable, each cleavage is regularly followed by the formation of new surface. Hence, it is impossible to accept Motomura's argument in the same chapter, that the failure of larvae in Ca-free sea water to develop into plutei is due more to the lack of new surface formation than to the falling apart of the blastomeres.

The assumption that the granules under discussion are located only in the cortical layer and not in the endoplasm has already been made by Motomura on the ground that exovates do not contain the granules. This conclusion is tenable only under the circumstance that the granules do not break down when the cells are injured, unlike the case with *Arbacia* granules. The authors' further experiments with this view in mind, together with their study of sectioned eggs, support Motomura's contention.

The authors are happy to acknowledge their indebtedness to the Marine Biological Laboratory of Woods Hole and to the Stazione Zoologica of Naples for the use of their research facilities.

Summary

1. In *Strongylocentrotus pulcherrimus* eggs, the granules seen in the living egg as orange-colored in white light and dark-red in light filtered through a Wratten No. 49 filter, and the large blackened granules seen in osmium-fixed sections, have been shown to be identical on the basis of size, position and behavior.

2. In unfertilized eggs, these granules are located directly beneath the surface, in which case it is difficult to distinguish them individually.

3. On fertilization, these granules migrate inward from the surface by 1,5 μ, leaving a finely granular zone of the same width at the cell periphery. This zone is called the " extra-granular zone " in the present paper.

4. During the process of segmentation, the equatorial surface of the uncleaved egg with the extra-granular zone and the granular layer forms the cleavage furrow without change in the mutual relations of the component parts except for the fact that the extra-granular zone on the polar side becomes thinner and the granules become more numerous on the sides of the furrow.

5. After the completion of cleavage, the accumulation of granules in the furrow disappears and the extra-granular zone on the polar side regains its pre-cleavage width—i.e., the distribution of the granules around the blastomeres momentarily becomes uniform.

6. The above stage is immediately followed by a phase of formation of new surface along the furrow region in which the extra-granular zone retreats from the sides of the furrow and the granules come into direct contact with the protoplasmic surface film. This arrangement on the new surface is the same as that of the unfertilized egg.

7. Kaolin particles adhering to the cell surface at the head of the cleavage furrow later come to lie at the border between the old surface (provided with the extra-granular zone) and the new surface (which lacks this zone). This is taken as an indication that the new surface is formed from the tip of the furrow, and that the protoplasmic surface film which is carrying the kaolin particles does not slip over the underlying cortex.

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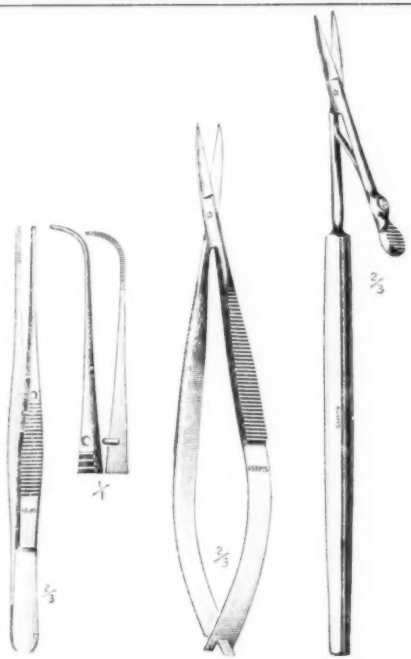

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